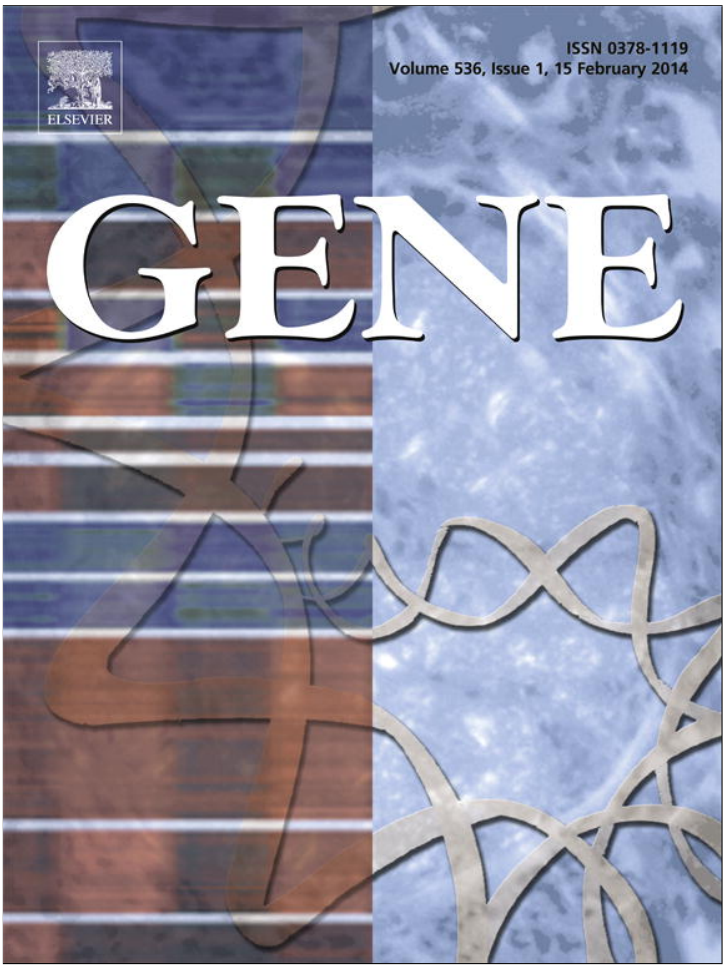

Gene 536 (2014) 29–39

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Role and importance of polymorphisms with respect to DNA methylation for the expression of CYP2E1 enzyme

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ARTICLE INFO

Article history:
Accepted 25 November 2013
Available online 12 December 2013

Keywords:
Hepatocellular carcinoma cell lines
CYP2E1 gene polymorphisms
DNA methylation
5-Azacytidine
Ethanol

ABSTRACT

Different individuals possess slightly different genetic information and show genetically-determined differences in several enzyme activities due to genetic variability. Following an integrated approach, we studied the polymorphisms and methylation of sites contained in the *5′ flanking region* of the metabolizing enzyme CYP2E1 in correlation to its expression in both tumor and non-neoplastic liver cell lines, since to date little is known about the influence of these (epi)genetic elements in basal conditions and under induction by the specific inductor and a demethylating agent. In treated cells, reduced DNA methylation, assessed both at genomic and gene level, was not consistently associated with the increase of enzyme expression. Interestingly, the Rsa/Pst haplotype differentially influenced CYP2E1 enzyme expression. In addition, regarding the Variable Number of Tandem Repeats polymorphism, cells with A4/A4 genotype showed a greater expression inhibition (ranging from 20% to 30%) compared with others carrying the A2/A2 one, while those cells bringing A2/A3 genotype showed an increase of expression (of 25%, about). Finally, we demonstrated for the first time that the A2 and A3 CYP2E1 alleles play a more important role in the expression of the enzyme, compared with other (epi)genetic factors, since they are binding sites for trans-acting proteins.

1. Introduction

Because of the genetic variability, different individuals possess slightly differing genetic information in their genomes for each gene; in particular, the CYP multigenic family, whose members code for xenobiotic-metabolizing enzymes, is one of the most polymorphic ones in humans. Consequently, different individuals may have different allelic variants of the same metabolizing gene and the resulting genotypes can endow each individual with a peculiarly-different metabolizing capability and, ultimately, distinct phenotypic metabolic behavior (Witkin et al., 2000). The phenotypic variation in the metabolism of many substances was proven to be the consequence of genetically-determined differences in the activities of a significant number of enzymes (Smith et al., 1995). For this reason it is interesting to study the gene expression of a metabolizing enzyme in correlation to anything that can bring to its diversification, in order to isolate and identify some environmental elements or lifestyles that can modify its normal function, causing a possible threat to human health.

The human enzyme CYP2E1 is a protein of about 62 kDa consisting of 493 amino acid residues, involved in the metabolism of more than 80 hydrophobic toxic compounds and contributing to the activation of many pro-carcinogens and certain drugs towards highly reactive forms (Guengerich et al., 1991). In particular the enzyme CYP2E1 activates the N-nitrosamines contained in tobacco cigarettes and in food (Wang et al., 2002), as well as in many carcinogens endogenous and/or of industrial origin (Agundez, 2004). It is also able to reduce the oxygen in highly reactive species, Reactive Oxygen Species (ROS), such as the superoxide anion O^{-}, the singlet oxygen ^{1}O, hydrogen peroxide H_2O_2 and hydroxyl radicals $OH^{\cdot}$ determining multiple effects in cells such as the denaturation of the proteins, the inactivation of enzymes and DNA mutations (Lu and Cederbaum, 2008).

The human *CYP2E1* gene, located in 10q24.3 (Piao et al., 2003) is induced by high levels of ethanol and its expression can be regulated at various levels: transcriptional, translational, mRNA stabilization and protein degradation (Lieber, 1999). Some Restriction Fragment Length Polymorphisms (RFLP) and Variable Number of Tandem Repeats (VNTR), contained in its *5′ flanking region* (Fig. 1), are relatively known: a RFLP for enzymes RsaI and PstI generating two variants, Rsa +/Pst− (named C1 haplotype) and Rsa−/Pst+ (named C2 haplotype), which are in complete *linkage disequilibrium* (Chao et al., 1995)

Abbreviations: CYP2E1, cytochrome p450 2E1; DNA, deoxyribonucleic acid; VNTR, Variable Number of Tandem Repeats; CYP, cytochrome p450; ROS, Reactive Oxygen Species; RFLP, Restriction Fragment Length Polymorphism; PCR, Polymerase Chain Reaction; HCC, hepato-cellular carcinoma; EDTA, ethylene diamine tetraacetic acid; ZCyd, 5-azacytidine; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]; DMSO, dimethyl sulfoxyde; MSRE-PCR, Methylation Sensitive Restriction Endonuclease-Polymerase Chain Reaction; MeSAP-PCR, methylation sensitive arbitrarily primed-polymerase chain reaction; EMSA, Electrophoretic Mobility Shift Assay; DTT, dithiothreitol; PBS, phosphate buffered saline; BSA, bovine serum albumin; HRP, horse radish peroxidase.

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http://dx.doi.org/10.1016/j.gene.2013.11.097

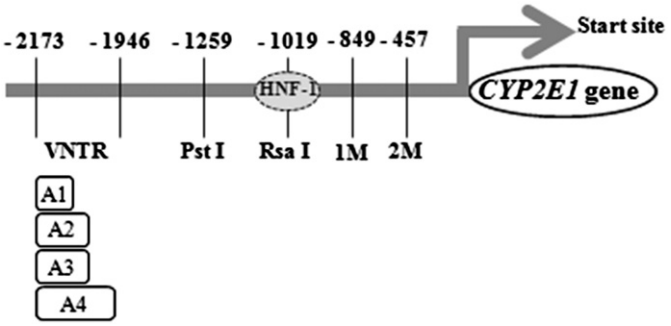

Fig. 1. Diagram of *5′ flanking region* of *CYP2E1* gene showing the position of VNTR region, Rsa/Pst polymorphism and methylation sites, with respect to start site. In dashed the reported position of HNF-1 transcriptional factor.

and VNTR, generating four allelic forms: A1, A2, A3, A4 (Itoga et al., 2001). In a previously published paper, our laboratory genotyped the CYP2E1 VNTR polymorphisms in a cohort of regionalized subjects, performing a Polymerase Chain Reaction-RFLP assay (PCR-RFLP), highlighting a possible association between two VNTR genotypes and the tendency to alcohol consumption and smoke, respectively (Catanzaro et al., 2012a).

To date, little is known about the regulation of this gene, the influence of polymorphisms in its expression and even less on methylation of potential methylation sites (Botto et al., 1994; Hu et al., 1995) (Fig. 1) as epigenetic regulatory elements. For this reason, we took up the challenge of an integrated approach: in this work, we associated epigenetic data with genetic polymorphism analysis (both RFLP and VNTR), analyzing the methylation status of the *CYP2E1 5′ flanking region*, assessing enzyme expression in both untreated cells and treated with the main inductor, ethanol, and a demethylating molecule, 5-azacytidine. Finally, on the basis of the data obtained, we hypothesized and demonstrated for the first time that the VNTR polymorphisms of the *CYP2E1* gene have a more important role in the regulation of the enzyme compared to other genetic and epigenetic factors being the binding site for trans-agent molecules that modulate the expression of the enzyme. We chose to conduct these studies on liver cell lines, both tumor and non-neoplastic, to mimic the metabolic condition of the main tissue where the CYP2E1 enzyme is functionally active.

2. Material and methods

2.1. Cell cultures and reagents

Hepato-cellular carcinoma (HCC) cell lines, Ha22T, HepG2, HuH7 and Chang liver cells, were routinely cultured as previously reported (Catanzaro et al., 2012b). Briefly, 1:1 DMEM-F12 (Invitrogen, USA), supplemented with 10% fetal calf serum (Gibco, Invitrogen, Carlsbad, USA), penicillin (100 U/ml) and streptomycin (100 μg/ml) was utilized as culture medium. Cultures were maintained at 37 °C in a 5% CO_2 humidified incubator. Cells grown were harvested by trypsinization (0.25% Trypsin with EDTA 4Na). Trypsin was therefore inactivated by the addition of the growth medium.

Ethanol 100% (Carlo Erba, Italy) and 5-azacytidine (ZCyd, Sigma, St. Louis) were micro-filtered with 0.20 μm filter (Sartorius, Germany) before dissolving in cultured medium. The doubling time of these cells was ranging from 22 to 24 h.

2.2. Cell viability in untreated and exposed cells

In order to determine the best time and dose of ethanol and ZCyd exposure, cell viability by Trypan Blue exclusion test was performed. This test was carried out in a time course ranging from 24 h to 48 h of increasing concentrations of drugs: the best results were found using ethanol exposure (10, 25, 50 or 100 mM) or ZCyd treatment (1, 2.5, 5 or 10 μM) for 48 h.

In detail, cells were washed twice with Hanks' Balanced Salt Solution and then harvested by trypsinization. 0.1 ml of cell suspension was mixed 1:1 with 0.4% Trypan Blue Stain (Invitrogen, USA), maintained for 5 min at room temperature; finally cells were counted in a Bürker chamber under light microscope. The percentage of viable cells was determined dividing the total number of viable cells *per* ml of aliquot and the total number of cells in the same volume (×100).

Regarding viability after treatments with 5-azacytidine (ZCyd), MTT assay was also performed. Approximately 5×10^3 cells of each cell line were seeded in a 96 wells cell culture plates (P96) for at least 18 h and subsequently treated with ZCyd (1, 2.5, 5, 10 μM) for a time course ranging from 24 to 48 h. At the end of the treatment the ZCyd was appropriately removed and fresh culture medium (DMEM) and MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) were added in a ratio of 1:25 to each well. After treatment with MTT, the plate was put in the dark and under stirring, at room temperature, for 5 min and subsequently incubated at 37°°C in a 5% CO for 1 h. After incubation, the P96 was centrifuged at 1800 rpm for 10 min, the supernatant was discarded and dimethyl sulfoxide (DMSO) was added, in order to solubilize the formazan crystals, and the P96 was dark-incubated for 15 min at room temperature. The absorbance read in a luminometer ($\lambda = 560$ nm) allowed us to determine the percentage of cell viability.

Using both untreated and treated cells, at least three independent experiments were carried out for all the dose–response assays; data were calculated as mean ± SD.

2.3. DNA extraction

Briefly, about 1×10^6 cells were seeded in 10 mm tissue culture dishes; after 18 h cells were (or not) exposed to ethanol or ZCyd for 48 h. At the end of the exposure the cells were washed twice with Hanks' Balanced Salt Solution and then harvested by trypsinization, re-suspended in fresh medium and finally pelleted by centrifugation at 12,000 ×*g* for 10 min. Supernatant was carefully removed without disturbing the pellet, then DNA from HCC cell lines and Chang liver, was obtained using PureLink™ Genomic DNA Mini Kit according to the manufacturer's protocol (Invitrogen, USA). The purified DNA was quantified and stored at −20 °C.

2.4. RsaI/PstI polymorphism genotyping

A RFLP-PCR was used to genotype CYP2E1 RsaI/PstI polymorphism. We set up the method previously described by Gao et al. (2007) with some modifications developed in our laboratory. Briefly, PCR was performed using a final volume of 50 μl containing 150 ng of genomic DNA, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 0.05 U/μl Taq polymerase (Invitrogen, USA) and 0.5 μM of each primer (5′-CCAGTCGAGTCTACAT TGTCA-3′sense and 5′-TTCATTCTGTCTTCTAACTGG-3′ anti-sense). PCR was carried out by 5 min at 95 °C for initial melting and subsequent 30 thermal cycles under the following condition: 1 min at 55 °C for primer annealing and 1 min at 72 °C for primer extension.

The PCR-amplified product is a 410 bp fragment of the transcriptional regulation region of the gene CYP2E1. Half of the total amount of the PCR product was separately subjected to enzyme restriction with Rsa I and Pst I (0.5 U/μl) in a final volume of 10 μl. To determine the genotype, digested and non-digested samples were analyzed by a 12% polyacrylamide gel stained with SYBR Safe DNA gel (Invitrogen, USA) and visualized by ChemiDoc XRS (Bio Rad, USA).

2.5. VNTR polymorphism genotyping

To genotype CYP2E1 VNTR polymorphism, we carried out the specific PCR-technique performed in our laboratory and described elsewhere

(Catanzaro et al., 2012a). This technique consists in a conventional PCR with specific primers (Itoga et al., 2002) and a subsequent restriction of the PCR product with Nla IV endonuclease which is able to give a digestion pattern specific for each VNTR genotype.

2.6. DNA methylation analysis

Methylation Sensitive Restriction Endonuclease-PCR (MSRE-PCR) (Longo et al., 2013) was performed in order to determine the methylation status of the sites CpG rich, present in the *5′ flanking region* (−849 bp and −457 bp to the start site, see Fig. 1) of the *CYP2E1* gene. In a previous paper we made a distinction among three kinds of cytosine, each potentially methylation site: the cytosine "CpG", "CHH" or "CHG", where H are all nucleotides except G (Bellavia et al., 2013). In the region described by Hu et al. (1995), containing the potential methylation sites of *CYP2E1* gene not canonically attributable to CpG island, there were two CpG sites present, specifically detected by isoschizomers HpaII and MspI (Biolabs, Europe), since the other sites are CHH or CHG. We focused our investigation on these two CpG sites whose methylation could be revealed by MSRE-PCR. We named these sites 1M and 2M, respectively.

For each sample two independent digestions with the isoschizomers were carried out over night at 37 °C; typically 150 ng of DNA and 1 U of the enzyme were used. At the end of the incubation, the digested products were amplified using 0.5 M each primers (5′-TGC AAC CTA TGA ATT AAG AAC-3′ sense and 5′-TAA AAG GAA AGA GTG AGT CAA-3′antisense for 1M and 5′-GAT GTT GAA TTT TCC TTC TGG-3′ sense and 5′-ATG GAG TTG GAC TGT GTC AAC-3′antisense for 2M), 0.2 mM each dNTP, 1.5 mM $MgCl_2$ and 0.05 U/μl of Taq (Invitrogen, USA). To assess the genome-wide DNA methylation status of the cell lines, we performed Methylation Sensitive Arbitrarily Primed-PCR (MeSAP-PCR), as previously described (Caradonna et al., 2007). Since ethanol has a specific effect on the CYP2E1 gene, and Cyd has a more generalized genomic effect, we chose to investigate the genomic changes of DNA methylation in ZCyd-treated cells, only.

Briefly, this technique consists in an arbitrarily primed PCR amplification, in which genomic DNA, digested with a couple of endonuclease (one of which being methylation sensitive), was used as template. The DNA fingerprinting, obtained from Polyacrylamide gel electrophoresis of the PCR amplifications, was subjected to a densitometer scanning using the software SigmaGel (Jandel Scientific, USA), by which it was possible to count the number of disappeared, appeared, intensified or attenuated bands. By these findings it is possible to have a measure of the ZCyd treatment-induced changes in global DNA methylation, particularly with regard to the CpG-rich sequences, as the used primer possessed a 3′ tail "CGCG". Statistical analysis was done using Student's *t* test comparing every HCC cell line to Chang liver cells; a value of at least $p < 0.05$ was considered as significant.

2.7. Determination of CYP2E1 enzyme expression by Western blotting

Western blot analyses were performed as previously reported (Catanzaro et al., 2010).

The detection of the chemiluminescent signal was obtained by Chemidoc XRS (Bio-Rad, USA) treating the membrane with SuperSignal West Femto Chemiluminescent Substrates according to the manufacturer (Thermo Scientific, USA).

The normalized expression of CYP2E1 enzyme (β-tubulin, Santa Cruz Biotechnology, Europe) was reported as histogram, derived from densitometric scanning, using the software Sigma gel (Jandel Scientific, USA), of the bands obtained by at least three Western blotting analysis. Each expression variation of a treated cell line was compared with its respective untreated. *p* value was calculated according to the χ^2 test and a value of $p < 0.05$ was considered significant.

2.8. Cloning

To ensure a greater specificity in the EMSA, described below, we chose to use fragments (containing the VNTR region of the *CYP2E1* gene) cloned in *Escherichia coli* from previous CYP2E1 PCR product, properly checked for compliance to the restriction map and sequenced. All amplified fragments (VNTR variants) were fractionated using the modified Voltage Gradient Gel Electrophoresis (VGGE) technique (Bellavia et al., 2004) and then eluted as described elsewhere (Barbieri et al., 1996). Eluted Amplicons were cloned in TOPO-TA plasmid, using the TOPO-TA Cloning Kit, according to manufacturer's (Invitrogen™, USA) specifications. All those colonies that resulted positive to *colony PCR*, carried out using a pair of specific primers (T3: 5′ ATTAACCCTCAC TAAAGGGA 3′; T7: 5′ TAATACGACTCACTATAGGG 3′), were initiated to growth for 12 h at 37° C in LB with ampicillin and tetracycline. Then, the plasmid DNA was extracted with the PureLink Mini prep kit according to the manufacturer (Invitrogen™, USA), quantified and controlled by serial reactions with restriction enzymes and sequenced to specifically confirm the real presence of the insert of our interest.

2.9. Electrophoretic Mobility Shift Assay (EMSA)

Previously cloned CYP2E1 A2 and A3 VNTR alleles, which are suspected to contain a protein binding sequence, were amplified and labeled with biotin (Invitrogen, USA). Separately, without biotin labeling, we obtained other cloned fragments as competitors or control: in particular, we used the same A2 and A3 alleles as specific competitors; we amplified a region of CDK2NA gene as a non-specific competitor and, finally we utilized A4 allele as a negative control, since we deducted that it didn't contain a specific protein binding sequence.

All PCR reactions were prepared on the basis of the same protocol: 30 ng di DNA template; 1× buffer (10 mM K-phosphate buffer pH 7, 100 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.01% Tween 20, 50% glycerol (v/v)); dNTP 200 μM; MgCl2 1.5 mM; primers 1 pmol/μl; Taq 0.025 U/μl (Invitrogen, Europe), in Thermalcycler PerkinElmer 9600, using the following specific primer pairs (Catanzaro et al., 2012a; Dehghan et al., 2008) in an appropriate PCR program: 1 cycle of denaturation 94 °C × 5′ and 30 cycle of denaturation 94 °C × 30″, annealing 60 °C × 30″, extension 72 °C × 30″; 1 cycle of final extension 72 °C × 5′ was also carried out.

Both DNA fragments and Chang liver nuclear protein extracts were incubated for 30 min at room temperature in 1.5 ml of the binding solution (150 mM TRIS–HCl pH 7.9–100 mM KCl-10 mM DTT). Then, the tubes, caps open, were exposed to UV light for about 30 min, to carry out cross-link between the DNA fragments and the protein(s). The samples were loaded onto a horizontal 12% acrylamide/bisacrylamide gel (29:1 ratio) under non-denaturing conditions and at 300 V (Izzo et al., 2006).

For blotting, a methanol-activated membrane (PVDF Membranes, GE Healthcare, USA) incubated for 10 min in distilled H O and 15 min in Transfer Buffer 1× [100 ml Transfer Buffer 10× (30.3 g Tris base, 144 g glycine, ddH_2O up to the volume of 1 L), 800 ml ddH O, 100 ml methanol] was used. Separately, the gel with resolved proteins was incubated in transfer buffer 1× for 15 min. Then, the sandwich for blotting was composed and the electro-blotting with 400 mA constant current was carried out for 1 h at 4 °C.

After the full transfer, the membrane was incubated (with the side of the complex DNA-protein facing upwards) in a denaturing solution (0.4 M NaOH–1.5 M NaCl) for 20 min, subsequently in a neutralizing solution (0.5 M Tris pH 7.5, 1.5 M NaCl) for 20 min. and finally in an equilibration solution (2× SSC) for 20 min. Then, the membrane was exposed for 2′ of UV light to perform the cross-linking between the membrane and DNA protein complex. Successively, the membrane was incubated for 1 h in hybridization solution (PBS 1×-Tween20 0.1%–5% free fatty acid milk), washed twice for 5 min each in PBS 1×-Tween20 0.1% and finally incubated overnight, on a tilting table, at

4 °C, in 10 ml of PBS 1×-Tween20 0.1%–3% BSA with 1:200 anti-biotin monoclonal antibody (Roche, Europe). Then, the membrane was washed three times, 10 min each, with PBS 1×-Tween20 0.1% and incubated at room temperature with HRP-conjugated anti-rabbit IgG diluted in 10 ml of PBS 1×-Tween20 0.1%–5% free fatty acid milk, for 1 h on a tilting table. Lastly, three washes with PBS 1×-Tween20 0.1% for 10 min were performed.

The detection of the chemoluminescent signal was obtained by Chemidoc XRS (Bio-Rad, USA) treating the membrane with SuperSignal West Femto Chemoluminescent Substrates according to the manufacturer (Thermo Scientific, USA).

3. Results

3.1. Cell viability

In order to determine whether the treatments induced a reduction of viability in hepato-cellular carcinoma (HCC) cell lines (Ha22t, Huh7, HepG2) and in Chang liver cells, Trypan blue exclusion and MTT test were used. Moreover, the cell viability assay determined the choice of the best time and dose of exposure with ethanol and ZCyd in order to exclude any lethal effects and/or clonal selection. In addition, since it is well known that ZCyd action is more evident after the cell division, the time of treatment took into account the doubling time of the cells allowing them to accomplish at least two cell cycles.

Under 48 h treatment at determined doses of ethanol (10, 25, 50 or 100 mM) and ZCyd (1, 2.5, 5 or 10 μM), viability was always over LD50 in the three HCC cell lines and in Chang liver cells (data not shown).

3.2. CYP2E1 genotyping

RsaI/PstI and VNTR CYP2E1 genotypes were determined; Table 1 shows the obtained results. Regarding CYP2E1 Rsa/Pst polymorphism, both Ha22T and HUH7 and Chang Liver showed the same genotype (R+−/P−−) while HepG2 showed a different one (R++/P+−). It is interesting to notice that all the four cell lines presented a non-canonical genotype association, attributable neither to C1 nor to C2 haplotype. Instead, regarding CYP2E1 VNTR polymorphism, only HepG2 and HUH7 presented the same genotype (A4/A4) while Chang liver and Ha22T exhibited A2/A2 and A2/A3 genotypes respectively.

3.3. DNA methylation analysis

Methylation status of the two sites named 1M and 2M (−849 and −457 bp from the start site, respectively) was assessed by MSRE-PCR both in untreated and in ethanol- or ZCyd-treated cells. Results are reported in Fig. 2.

Both untreated HCC lines and Chang liver showed a hypermethylation of the two sites, 1M and 2M, with the exception of the 2M site in the HepG2 cells which appeared partially methylated. The response to ethanol or ZCyd exposure was different among the four cell lines: HepG2 cells seem to have the 2M site quick to demethylation in relation to exposure, more to ethanol than to ZCyd, Chang Liver cells instead showed a severe resistance to demethylation in both the sites and in relation to every exposure.

Table 1
Rsa/Pst and VNTR CYP2E1 genotypes of HCC lines and Chang Liver cells.

Cell lines	CYP2E1 polymorphisms	
	Rsa/Pst	VNTR
Chang liver	R+−/P−−	A2/A2
Ha22T	R+−/P−−	A2/A3
HepG2	R++/P+−	A4/A4
HuH7	R+−/P−−	A4/A4

Genome-wide methylation status, investigated in untreated and ZCyd-treated cell lines, was analyzed by MeSAP-PCR. The results are reported in Fig. 3 and Table 2. As expected, the global methylation decreased in treated cells with respect to untreated ones; HCC cell lines exhibited less genomic DNA methylation both in basal condition even under ZCyd-treatment with respect to Chang Liver cells. In particular, HepG2 cells showed an elevated reduction of DNA methylation, as attested by the high number of DNA methylation variations significantly different from those of normal cells, according to Student's *t* test ($p < 0.05$).

3.4. CYP2E1 enzyme expression

In order to understand how 48 h treatments with ethanol or ZCyd can affect CYP2E1 enzyme expression through a variation of the DNA methylation of the *CYP2E1 5′ flanking region*, we carried out Western blot analyses using a polyclonal antibody against the CYP2E1 protein in a discriminated total protein extracts of the untreated and treated cell lines. The results are shown in Fig. 4.

In a first overall view, it is evident that ZCyd is more effective than ethanol at inducing CYP2E1 enzyme expression, but a more careful analysis shows that no cell line showed any dose-dependent behavior either under ethanol and ZCyd treatment. In particular, HepG2 cells have an ethanol-only related enzyme expression while almost all ZCyd-treated cell lines show a bell-trend enzyme expression, with an initial increase, a maximum, and a final decrease. All the expression variations of treated cells, compared with their respective untreated values, resulted not significant, according to χ^2 test.

3.5. Multiple correlation between ethanol- or ZCyd-inducted enzyme expression and CYP2E1 5′ flanking region DNA methylation

In order to evaluate whether and how changes in the methylation of *5′ flanking region* of the *CYP2E1* gene, induced by treatment with ethanol or with ZCyd, may affect the production of the enzyme, we correlated the results obtained from the study of DNA methylation of the two sites 1M and 2M with those regarding gene expression: essentially an overlap between the data presented in Figs. 2 and 4. In addition to what was previously said about the two phenomena separately considered, Fig. 5 demonstrated that there is no dose-dependent correlation between ethanol/ZCyd treatments, 1M and 2M site demethylation, and the enzyme expression.

3.6. Multiple correlation between ethanol or ZCyd-inducted CYP2E1 enzyme expression and CYP2E1 polymorphic genotypes

With the aim to correlate the effect of the polymorphic genotypes in the induction of enzyme expression, we correlated the enzyme intracellular amounts with possession of a certain polymorphic genotype, either in basal condition (untreated cells) or under 48 h treatment with ethanol or ZCyd. For this correlation, according to Wang et al. (2009) we chose the average concentration of ethanol (25 mM) and the average concentration of ZCyd (2.5 μM), since we know that it induces the highest increase of protein expression.

The correlation demonstrates that HepG2 and HUH7 cells had a lower basal enzyme expression, with respect to non-neoplastic cells; they showed the same homozygous VNTR genotype (A4/A4), differently from Chang liver and Ha22T cells. In the same graph it is possible to see that Ha22T showed a greater enzyme expression, with respect to control cells, having a heterozygous VNTR genotype (A2/A3). In general it can be observed that the presence of a VNTR allele with a higher number of repetitive units (in double dose) causes a decrease of enzyme expression (Fig. 6A). Surprisingly, genotypically-equal treated cells responded in different ways to ethanol or ZCyd induction (Fig. 6B): in particular, it is more evident looking at the most different enzyme expression in those cells having the same Rsa/Pst polymorphism (Chang

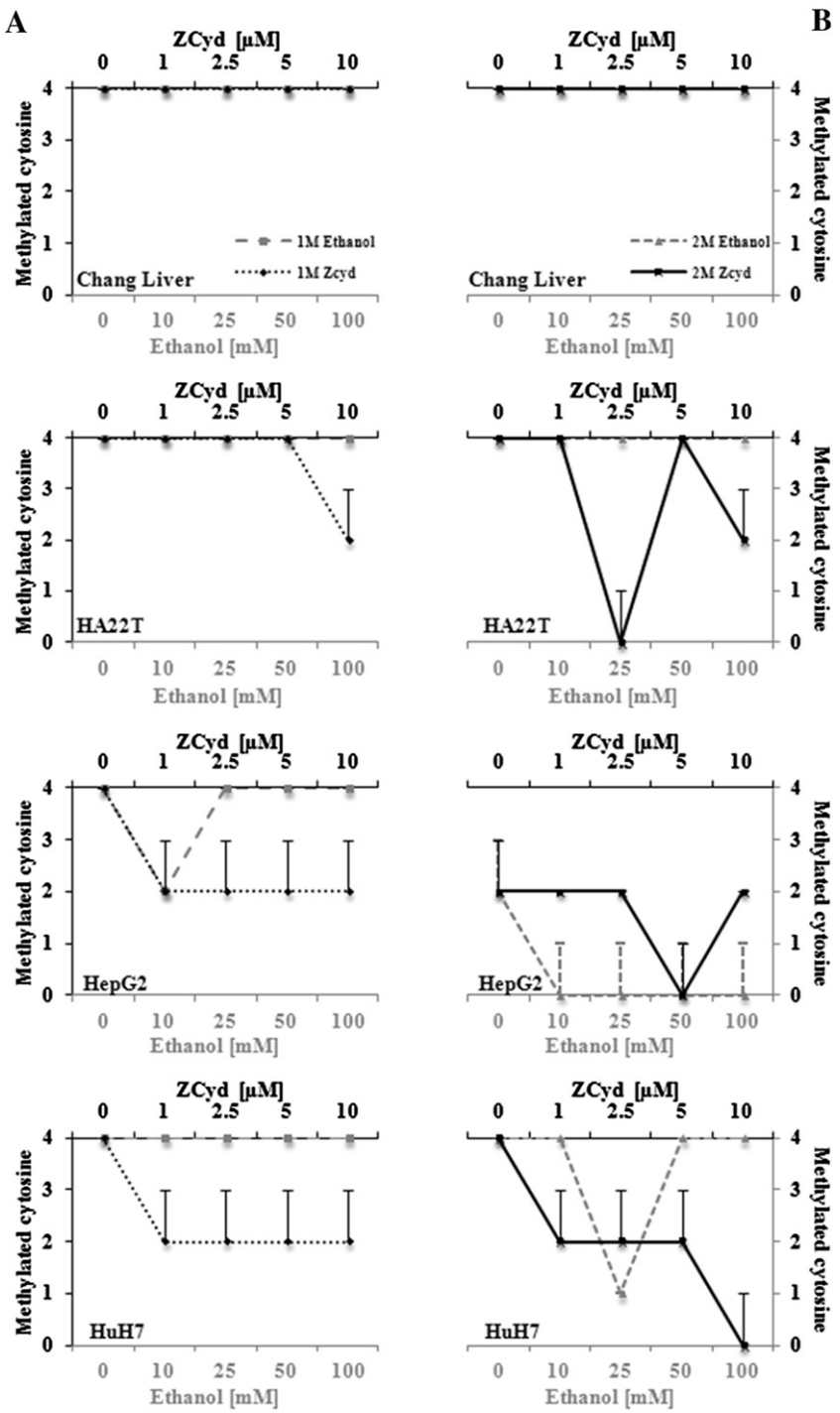

Fig. 2. Methylation status obtained by MSRE-PCR of the two sites 1M (A) and 2M (B) of the *5′-flanking region* of the *CYP2E1* gene after 48 h ethanol (gray) or ZCyd (black) exposure. Error bars represent the variable number of methylated cytosines obtained upon MspI enzyme methylation sensitivity.

liver, Ha22T and HuH7). Interestingly, looking at the ZCyd-treated cells and their VNTR genotypes, it seems that the general observation previously enunciated, regarding the presence of a VNTR allele with a higher number of repetitive units and decreasing of enzyme expression, is here specularly inverted.

3.7. VNTR allele-protein interactions studies

A2 and A3 VNTR alleles were investigated for their capabilities to interact with putative effector proteins, since those alleles, giving a higher CYP2E1 enzyme expression in untreated cells, leave us to assume that their action was mediated by trans-acting factors. Electrophoretic Mobility Shift Assay (EMSA) was performed onto *E. Coli*-cloned CYP2E1 VNTR fragments in the presence or absence of nuclear protein extracts of Chang Liver cells.

As it can be seen in Fig. 7A, both A2 and A3 VNTR alleles showed the electrophoretic mobility shift, when they were put in the conditions to bind specific cellular proteins. It is also important to note that these DNA–protein interaction can be defined “specific”, since mobility shift was not observed in the presence of a specific competitor (added in large excess and unlabeled) and, conversely, it was observed in the presence of large quantities of unlabeled unspecific competitor. This last finding was equally seen, as expected, in the presence of large quantities of unlabeled A4 allele, used as negative control, confirming that

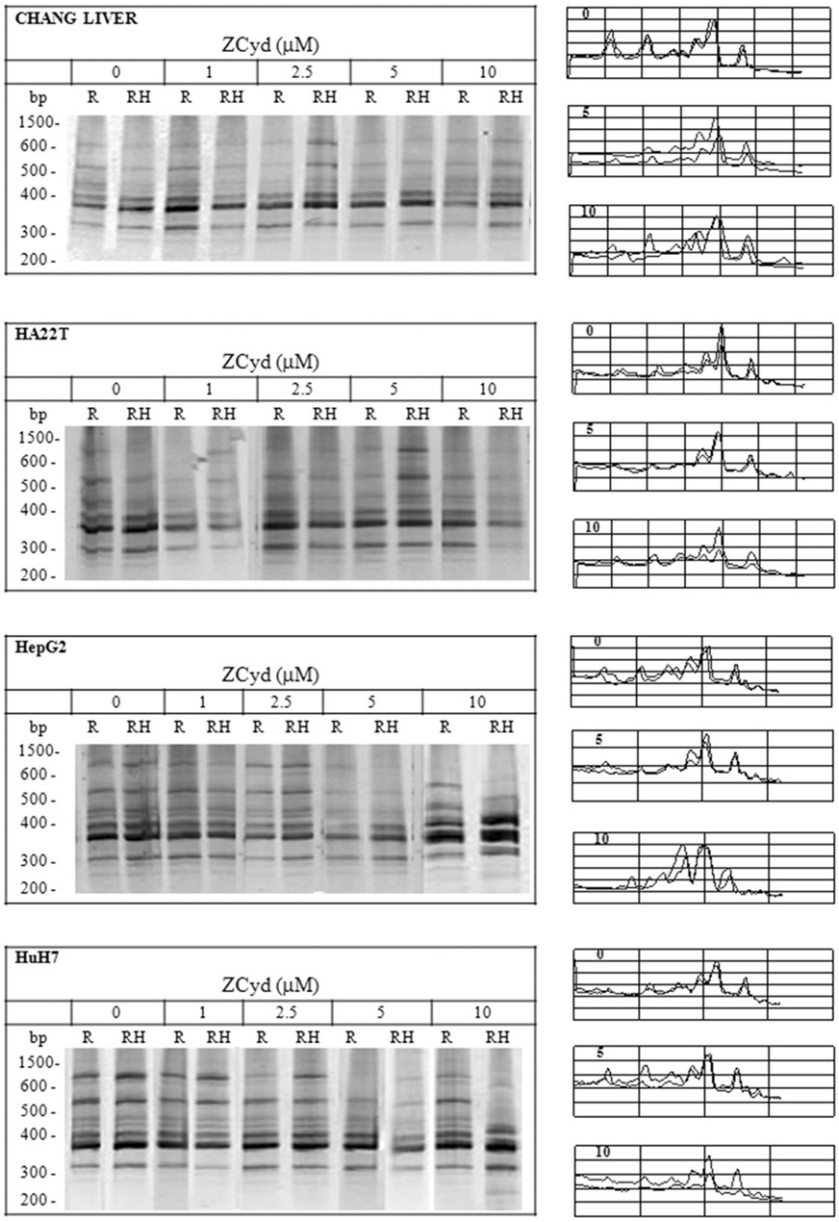

Fig. 3. DNA fingerprinting obtained by MeSAP-PCR (left) and corresponding densitometric scanning (right) of some representative experiments. R, RsaI single digested DNA; RH, RsaI/HpaII double digested DNA; bp, base pairs.

this large VNTR allele was not involved in any specific DNA–protein interaction.

Moreover, Fig. 7B shows the ethidium bromide-stained polyacrylamide gel conducted with the two VNTR alleles before the chemoluminescence detection. As it is clearly visible, the electrophoretic analysis shows a differential mobility shift of A2 with respect to A3 allele, indicating a different DNA–protein interaction among these two alleles.

4. Discussion

4.1. An integrated approach to study genetics and epigenetics of CYP2E1gene

The increasing interest of the scientific community for CYP genes is today due to the possible correlation between the CYP enzymes and the onset/maintenance of liver cancer and in particular, hepatocellular carcinoma. The study of the regulation of the *CYP2E1* gene is also timely since it is well known that an inducer of its expression is acute ethanol consumption, still rising, unfortunately, among the younger.

Therefore, clarifying the logical links among alcohol induction, the expression of the *CYP2E1* gene and liver tumors could be of crucial importance and would be a great contribution in the field of clinical/diagnostic as well as an aid in understanding and management of the social problem of alcoholism.

Few data regarding the regulation of gene expression of the gene CYP2E1 have been reported so far and insufficient results have been published concerning the CYP2E1 *5′ flanking region* in which are contained both length or sequence polymorphisms as well as sites of DNA methylation not canonically related to CpG islands (Hu et al., 1995).

The data presented here are framed in this problem and represent the first attempts to clarify the dynamics of CYP2E1 enzyme expression with an integrated approach, genetic and epigenetic, studying the genetic variation (polymorphisms) and the epigenetic variation (DNA methylation) in relation to the amount of the expressed protein product. This work aims to be a study of functional correlation (epi)genotype–phenotype that find its reason in the equivalent importance of genotypic variations, such as polymorphisms (Watanabe et al.,

Table 2
Genomic methylation changes observed in DNA fingerprintings of untreated and ZCyd-treated cell lines.

Cell Lines	ZCyd (μM)	Fragment pattern variation		Total of variations
		Appearing or disappearing	Attenuation or intensification	
Chang liver	0	0	1	1
	1	0	1	1
	2.5	1	0	1
	5	1	0	1
	10	5	0	5
	Σ	7	2	9
HAH7	0	1	3	4
	1	0	4	4
	2.5	1	5	6
	5	1	1	2
	10	0	6	6
	Σ	2	19	21
HepG2	0	3	3	6
	1	2	3	5
	2.5	2	7	9
	5	2	1	3
	10	4	1	5
	Σ	13	15	28*
HUH7	0	0	2	2
	1	1	3	4
	2.5	1	3	4
	5	2	1	3
	10	6	1	7
	Σ	10	10	20

* $0.01 < p < 0.05$, according to Student's *t* test.

1990), and epigenetic changes, such as differences in DNA methylation (Lund and Van Lohuizen, 2004), in the expression of every gene and this one, in particular.

The cell-system to which we have chosen to apply this approach is represented by four cell lines: a non-neoplastic cell line (Chang Liver) and three tumor cell lines (HA22T, HepG2 and HuH7). These cell lines, being of hepatic origin, can mimic in vitro genomic and metabolic conditions, since the CYP2E1 is a hepatic microsomal fraction enzyme (Chao et al., 1995).

4.2. CYP2E1 genotyping

Several studies described the presence of two sequence polymorphisms associated with restriction sites for RsaI and PstI restriction endonucleases in the 5′ *flanking region* of the *CYP2E1* gene (Watanabe et al., 1990). In particular, some studies indicated that these two points of polymorphism form a haplotype in complete *linkage disequilibrium* (Chao et al., 1995), although a discrepancy in the association of these two polymorphisms was described in African-American and Japanese populations with a frequency of 6.8% and 4%, respectively (Kato et al., 1992).

Our data explain that 3/4 cell lines show the same polymorphic haplotype (Rsa+−/Pst−−) but this finding does not involve the three tumor cell lines and therefore any association between this haplotype and the transformed phenotype cannot be directly assumed. Interestingly, it should be noted that all the cell lines had a non-canonical haplotype, attributable neither to C1 nor to C2 haplotype, suggesting the hypothesis that these uncommon particular haplotypes could constitute a selective advantage, favoring cell adaptation to in vitro growth. Additionally, as it happens in all the in vitro established cell lines, it is also possible that stabilization-induced/inducing chromosomal aberrations may have produced supernumerary copies of chromosome 10 or of 10q region. This can result in a generation of multiple copy number of *CYP2E1* gene which, towards a mutational divergence, could give a complex and non-canonical Rsa/Pst haplotype to the cell lines.

Little is known about the length-polymorphisms of *CYP2E1* gene: only two reports described these VNTR alleles (Itoga et al., 2001; Lee et al., 2008) and explained the composition of repeating units. The cell lines we genotyped had different VNTR genotypes, except HuH7 and HepG2 cells that showed both the A4/A4 genotype. More interesting correlations will be reported in this work about the role of these less-studied genetic variations and CYP2E1 enzyme expression.

4.3. DNA methylation analysis

In order to characterize the functional role of the methylation sites described in the promoter of the *CYP2E1* gene, we treated the four cell lines with increasing doses of ethanol and ZCyd for 48 h. It is worth noting that ethanol is a specific powerful positive modulator of *CYP2E1* gene expression (Tsutsumi et al., 1993) and that ZCyd is a cytidine analog which is able to reduce DNA methylation towards its inhibitory effect on the methyltransferase enzyme DNMTs, so as to be called "DNMT inhibitor of reference" (Ceccaldi et al., 2013).

Our working hypothesis predicted that using these two drugs in parallel could give more information, direct and indirect, about the CYP2E1 methylation site function. By treatment with a specific gene-inducer it would be possible to verify if the methylation sites must necessarily decrease to permit the enzyme expression. In contrast, treating the cells with a drug able to reduce DNA methylation in a non-specific way, it was possible to know whether, forcing the demethylation of sites, the gene was also expressed in the absence of its own specific inducer.

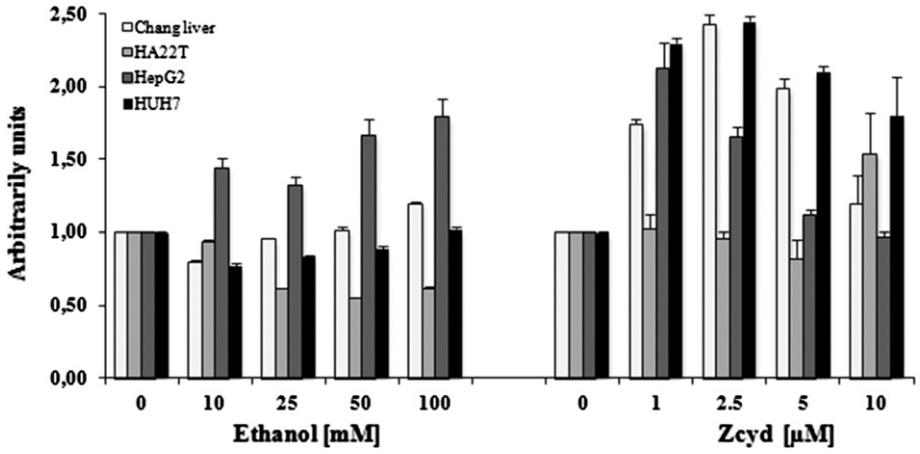

Fig. 4. Histograms indicating, in arbitrary units, the results of densitometer scanning of Western blotting signals of CYP2E1 enzyme expression obtained normalizing every (un)treated cell line expression against respective β-tubulin value and relating it with untreated control set as 1. Data were expressed as mean ± SD from three independent experiments.

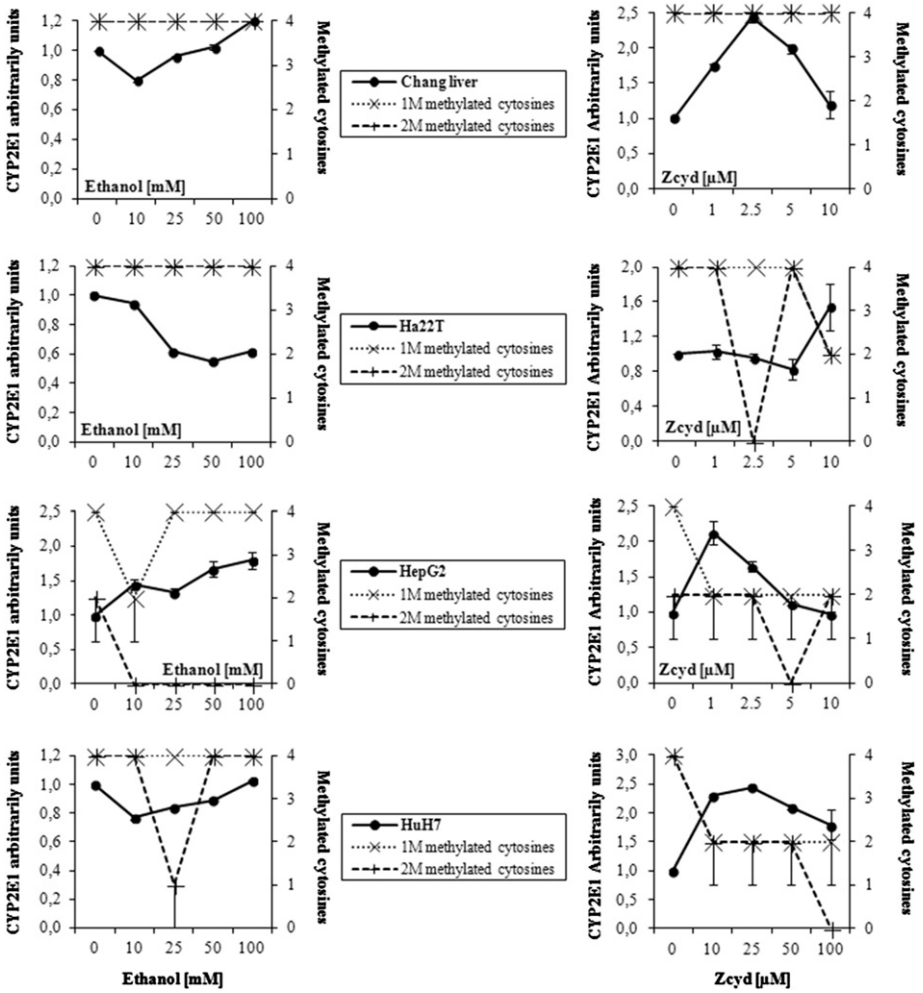

Fig. 5. Simultaneous graphic representation of ethanol- (left) or ZCyd- (right) inducted enzyme expression and DNA methylation status of the two sites 1M and 2M of the *CYP2E1 5′-flanking region* in the four cell lines. The enzyme expression is indicated in arbitrary units as the results of densitometer scanning of Western blotting signals normalized against respective β-tubulin value and related with untreated control set as 1; data were expressed as mean ± SD from three independent experiments. The DNA methylation data were obtained by MSRE-PCR and the relative error bars represent the variable number of methylated cytosines obtained upon MspI enzyme methylation sensitivity.

DNA methylation data of the gene in untreated cells showed that both sites in all cell lines, with the unique exception (2M site in HepG2 cells), are hypermethylated suggesting that this condition corresponds to a basic DNA methylation level of the gene in unstimulated cells. The DNA methylation analysis of the two sites (1M and 2M, Fig. 2) in ethanol- or ZCyd-treated cells showed no consistent results in the four cell lines. In particular, either increasing the ethanol dose or exposing to ZCyd escalating treatment, the two methylation sites showed a heterogeneous behavior. Looking at these results, it is difficult to glimpse for these two inductors a direct relation between

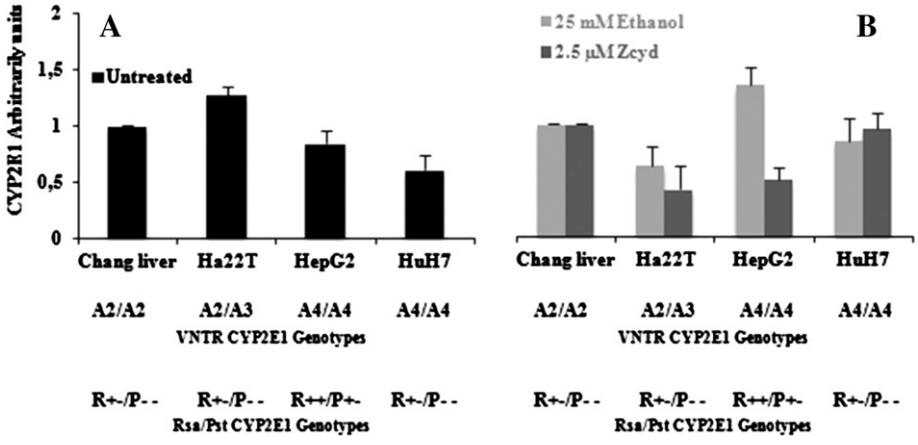

Fig. 6. Simultaneous graphic representation of untreated (A) and ethanol- or ZCyd- (B) induced enzyme expression and polymorphisms genotypes (reported below) in the hepatic cell lines. The enzyme expression is indicated in arbitrary units as the results of densitometer scanning of Western blotting signals normalized against respective β-tubulin value and related with untreated control cell line (Chang liver cells) set as 1; data were expressed as mean ± SD from three independent experiments.

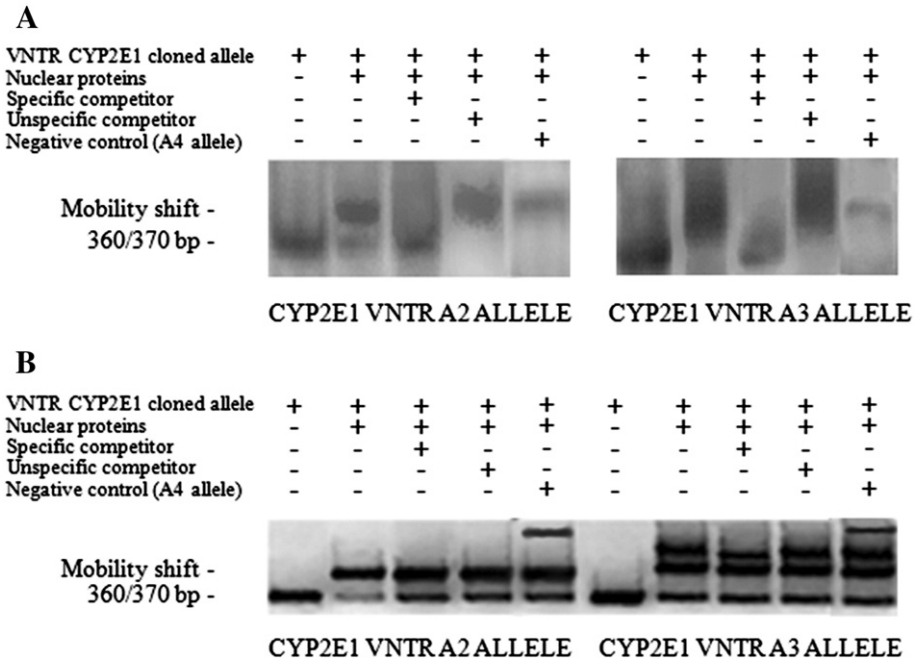

Fig. 7. Electrophoretic Mobility Shift Assay (EMSA) of cloned VNTR CYP2E1 A2 and A3 alleles put in presence of the Chang liver total protein extract. (A) Chemoluminescent detection. (B) Ethidium bromide-stained gel electrophoresis, catched before chemoluminescent detection.

doses–response in terms of treatment and demethylation of the 1M and 2M sites.

We could say that the two inducers, the first CYP2E1-specific and the second specific for all the DNA methylation-regulated genes, cause a general reduction of methylation at all two sites of cancer cell lines (HepG2, Huh7 and HA22T) but we have also to report that neither ethanol nor ZCyd is able to reduce, in any dose and time conditions, the CYP2E1 DNA methylation in the non-neoplastic Chang Liver cells. It is well known that the genomes of normal cells are globally more highly methylated than those of cancer cells (Gama-Sosa et al., 1983): within this scenario, our data confirm that, at least for the CYP2E1 promoter, the normal cells appear insensitive to the inducer-agent action.

Finally, seeing the data in cancer cells, it is possible to observe that the 2M site is overall more sensitive to the reduction of methylation rather than the site 1M; remembering that this site, between the two, is the closest to the start site, it would be possible to assign to the 2M site a more dynamic ability to modulate their methylation status in relation to external stimuli.

DNA methylation was investigated in all the four cell lines treated or not with ZCyd, at genome level too. Results (Fig. 3 and Table 2) of MeSAP-PCR were in agreement with those obtained by MSRE-PCR for *CYP2E1* gene, showing an overlap between genomic- and gene-level. A generalized decrease in DNA methylation was observed in ZCyd-treated cells compared to untreated and this behavior happened more in HCC cells than in Chang liver, confirming the well-known genomewide demethylating effect of ZCyd. In particular, the genome of HepG2 cells was found to be the most sensible to hypomethylation ZCyd-induced, showing significant differences ($p < 0.05$) in DNA methylation changes with respect to the untreated. Conversely, Chang liver cells exhibited their genome hypermethylated both in basal conditions and under ZCyd-treatment, as can be seen by the low number of methylation changes.

4.4. Multiple correlation between ethanol- or ZCyd-inducted enzyme expression and CYP2E1 5′ flanking region DNA methylation

Since the histograms showed in Fig. 4 failed to give us a clear relation between induction of ethanol or ZCyd and enzyme expression, we performed a multiple correlation between ethanol- or ZCyd-inducted enzyme expression and CYP2E1 *5′ flanking region* DNA methylation, carrying out an integrated approach of these data. The results (Fig. 5) are not easy to interpret: in fact, it is evident that both ethanol and ZCyd influenced the CYP2E1 enzyme expression but it is equally evident that this influence did not appear to follow the logical path of the increase of enzyme expression consistent to a reduction of methylation, ethanol- or ZCyd-inducted. However, the results suggest a complex control-mechanism with several actors (not yet all known), each of which, differently influenced by the reduction of methylation, induces the enzyme expression in its own way. Therefore, the regulation of the expression of the CYP2E1 enzyme is more complex than initially proposed (Köhler and Roos, 2008). Finally, these data confirm that, within the *5′ flanking region*, the two methylation sites behave differently suggesting that they play different roles and functions in controlling the enzyme expression.

4.5. Multiple correlation between ethanol or ZCyd-inducted CYP2E1 enzyme expression and CYP2E1 polymorphic genotypes

A second multiple correlation, according to the mentioned integrated approach, was performed between ethanol- or ZCyd-inducted CYP2E1 enzyme expression and *CYP2E1* polymorphic genotypes. We integrated the data of CYP2E1 enzyme expression in untreated cells with possessed polymorphic genotypes with the aim to assess the influence of these genetics elements in enzyme expression. In fact, it is important to consider not only the methylation status of the cytosines of promoter, in an exclusive epigenetic approach, but also to associate genetic data about particular polymorphic genotypes, which is known to confer different gene expression, equally to gene methylation (Witkin et al., 2000).

Several studies were conducted on this gene with regard to the presence of Restriction Fragment Length Polymorphisms (RFLP) for the enzymes RsaI and PstI in the CYP2E1 *5′ flanking region* (Watanabe et al., 1990), as well as the effects of these polymorphisms in the transcriptional activity of the gene (Hayashi et al., 1991).

An interesting finding reported in this study (Table 1 and Fig. 6) is that all the used cell lines had a similar non-canonical Rsa/Pst genotype association, and in particular, that the HepG2 cells possessed an extra RsaI site with respect to the other cell lines; the Rsa I polymorphism is known to be particularly important for the transcriptional activity of

the *CYP2E1* gene, since it is a binding site of HNF-1 factor (Fig. 1) to the promoter (Watanabe et al., 1995). A single nucleotide change in this position alters the binding profile of HNF-1 resulting in a marked change in the transcriptional activation of gene.

Since these cell lines showed a similar Rsa/Pst polymorphic genotype, we were able to interpret the enzyme expression as resulting from the contribution of VNTR genotype only. Little is known about the relation between VNTR CYP2E1 polymorphisms and their influence in enzyme expression. Uchimoto et al. (2007) reported that these polymorphisms are involved in the negative regulation of transcription of the *CYP2E1* gene. The data reported here (Fig. 6A) show the greater inhibitory effect of A4/A4 genotype (containing more repetitive units), compared with the A2/A2 (containing less repetitive units); furthermore, our data firstly provide a quantitative estimation of the negative influence of the A4/A4 genotype with respect to A2/A2 in the enzyme expression. In fact, untreated HepG2 and HUH7 cells, carrying both A4/A4 genotype, showed an enzyme expression of less than or about 20% and 30% respectively, compared to untreated Chang Liver cells, and this small difference could be derived by the Rsa/Pst genotype, having HepG2 as an extra RsaI site. Similarly, we are able to describe for the first time the positive influence of the A2/A3 genotype in CYP2E1 expression: untreated HA22T cells, carrying A2/A3 genotype, showed about 25% fold increased of enzyme expression in comparison to the reference cells. These results, obtained from a panel of four cell lines, homogenous for the tissue origin, phenotypically different, being tumor and non-neoplastic cell line, were sufficient to let us to formulate a reasonable explanation, and to suppose some hypothetical roles and actors. We think that the expression of the enzyme could be mainly influenced by the polymorphic genotypes, more by VNTR rather than Rsa/Pst. These differences, together with hypothetical additional DNA methylation-sensitive regulatory elements, as well as *post*-transcriptional and *post*-translational mechanisms, could explain the inconsistent epigenetic behaviors previously showed.

Soichot et al. (2011) reported that VNTR sequences in the promoter of the *IDO1* gene are binding site for trans-acting factors and that the occurred binding mitigates the VNTR inhibitory effect on gene expression. In this scenario, we considered reasonable to investigate whether the *CYP2E1* VNTR polymorphisms may also be a binding site for trans-acting factors, expressed by other genes, and whether this association may modulate the inhibitory effect of VNTR repeats onto *CYP2E1* gene expression. Whereas the increase of the enzyme expression cannot be ascribed to the demethylation of CYP2E1 sites or to the Rsa/Pst polymorphisms, we formulate the work-hypothesis that this increase was caused by the demethylation, ZCyd-induced, of promoters with consequent expression of other genes that encode for various *CYP2E1* effector proteins, either inhibiting or activating the *CYP2E1* gene expression. These effectors, taking a selective contact with each VNTR region, could act differently depending on their own nature and of VNTR allele length they bind to. In particular, the largest VNTR A4 allele didn't bind any protein(s), then, with respect to the untreated cells, a decreased expression of CYP2E1 gene occurred, especially in the presence of an A4/A4 homozygous genotype and two Rsa I sites (as it was observed in HepG2 cells, Fig. 6B). An inhibitory effect was conversely evident in the presence of shorter A3 allele, as it was seen in Ha22T cells. Surprisingly HUH7 cells, having the same A4/A4 genotype of HepG2, showed a ZCyd-induced increase of enzyme expression. It is worth mentioning that these cells had with respect to HepG2, a different haplotype Rsa/Pst for the presence of only one Rsa I sites.

In a previous paper we hypothesized that the A4/A4 genotype may be a high-risk genotype during the early stages of cancer (Catanzaro et al., 2012a) and in particular for pancreatic adenocarcinoma. In light of the data showed here, since it is well known that the genome of a tumor cell is globally demethylated (as that of a ZCyd-treated cell) we confirmed our previous hypothesis and launch the idea that having the A4/A4 genotype for a tumor cell is a selective advantage, especially when the same cell is gradually demethylating its own genome.

All the data referred here regarding ethanol treatment (Fig. 6B) seem to be confusing and often not in agreement with the others. In a recent paper, Balusikova and Kovar (2013) demonstrated, just in HepG2 cells, that only the long term exposure of ethanol can give an effect on CYP2E1 expression: thus, it is possible that the lack of clarity in the results reported here is in relationship to the short time of ethanol exposure we used. On the contrary, we think that our apparently inconsistent results, obtained using ethanol, are a confirmation of hypothesis regarding the ZCyd induction of other gene coding for *CYP2E1* effector proteins. In fact, as it is well known, ethanol is a specific inducer of *CYP2E1* gene and, unlike ZCyd, cannot act on other genes.

4.6. VNTR allele–protein interactions studies

In order to confirm or discard the hypothesis that VNTR polymorphisms are binding sites for trans-acting factors that can modulate the expression of the CYP2E1 enzyme, the Electrophoretic Mobility Shift Assays (EMSA) was carried out for the alleles A2 and A3, which were seen to be associated with an increased expression of the CYP2E1 enzyme in the untreated cells Ha22T carrying the two alleles. Thus, amplified VNTR regions were put in the presence of nuclear protein extract to differentiate the electrophoretic mobility of the DNA–protein complex compared to boundless DNA. A series of special features, either in the design and in the execution phase, preceded the planning and execution of the EMSA test. Firstly, we cloned the amplified fragments containing the VNTR alleles in plasmid vector and performed EMSA with PCR products obtained from amplification of specific VNTR clones, verified both with restriction analysis and sequencing: this in order to avoid unspecific results, strongly probable if it was chosen to use amplified repetitive regions (such as VNTR) obtained directly from genomic DNA. Finally, for each allele, we planned to repeat the test in the presence of specific, unspecific competitors and, more importantly, using a large amount of unlabeled VNTR A4 allele as negative control, since it must not bind any specific protein, according to our hypothesis. All these precautions made the assay able to discriminate specific DNA–protein binding complex.

By looking at the results obtained (Fig. 7A), it is evident that the *CYP2E1* VNTR alleles A2 and A3 possess binding sites for proteins and also that they are specific for these DNA fragments. Furthermore, as it is clear by looking at the ethidium bromide-stained gel (Fig. 7B), the differential mobility shift displayed between the two alleles showed that the related factors are different for the two tested alleles and this could justify their dissimilar influence in enzyme expression (Fig. 6A).

This study, demonstrating for the first time that the VNTR alleles A2 and A3 of the *CYP2E1* gene bind specific proteins, contributes to define a hypothetical model of regulation of the expression of this enzyme. It will be necessary to characterize the protein binding the VNTR alleles to clarify the complex regulation of CYP2E1 enzyme expression, either by genetic or epigenetic elements, to give useful topics in pharmacogenomics, for typing people regarding its metabolizing capability and therapy response, in oncology, for the diagnosis and therapy of hepatocellular carcinoma, or in the social, for the relation between *CYP2E1* gene and alcoholism.

Conflict of interest

All the authors state that there is any actual or potential conflict of interest.

Acknowledgments

This work was mainly supported by a grant from the University of Palermo (ex 60% and FFR). The authors are grateful to Prof. Claudio Luparello and to Dr. Alberto Sposito.

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