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Functional genomics and SNP analysis of human genes encoding proline metabolic enzymes

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Abstract

Proline metabolism in mammals involves two other amino acids, glutamate and ornithine, and five enzymatic activities, Δ^1 -pyrroline-5-carboxylate (P5C) reductase (P5CR), proline oxidase, P5C dehydrogenase, P5C synthase and ornithine- δ -aminotransferase (OAT). With the exception of OAT, which catalyzes a reversible reaction, the other 4 enzymes are unidirectional, suggesting that proline metabolism is purpose-driven, tightly regulated, and compartmentalized. In addition, this tri-amino-acid system also links with three other pivotal metabolic systems, namely the TCA cycle, urea cycle, and pentose phosphate pathway. Abnormalities in proline metabolism are relevant in several diseases: six monogenic inborn errors involving metabolism and/or transport of proline and its immediate metabolites have been described. Recent advances in the Human Genome Project, *in silico* database mining techniques, and research in dissecting the molecular basis of proline metabolism prompted us to utilize functional genomic approaches to analyze human genes which encode proline metabolic enzymes in the context of gene structure, regulation of gene expression, mRNA variants, protein isoforms, and single nucleotide polymorphisms.

Keywords

Apoptosis; FASTSNP; Functional genomics; OAT; OH-POX; OMIM; P53; Δ^1 -pyrroline-5-carboxylate (P5C); P5CDH; P5CR/PYCR; P5CS/PYCS; POX/PRODH; L-Proline; Promoter analysis; SNP

Introduction

With the addition of two new members, selenocysteine (Bock et al., 1991) and pyrrolysine (Hao et al., 2002; Srinivasan et al., 2002), now there are 22 known natural, genetically encoded, proteingenic amino acids in living organisms. Proline, one of the 22 proteingenic amino acids, is traditionally categorized as one of the nonessential amino acids in mammals because there is a specific set of enzymes designated to synthesize proline endogenously from its precursors in cells. However, it becomes evident that proline is conditionally indispensable in certain physiological conditions and cell types during mammalian development, in certain cells of the neonatal small intestine (Reeds 2000, Wu and Knabe, 1995). The metabolic pathways concerning proline have been observed to be very unique and multifunctional. These mostly mitochondria-based pathways involved in the biosynthesis and degradation of proline interact with the urea cycle, pentose phosphate pathway, and TCA cycle, not to mention the currently

discovered relationship between the metabolites and cell homeostasis (Phang et al., 2001, Hu et al., this issue, Phang et al., this issue).

Five enzymatic activities/reactions catalyze the interconversions of proline, glutamate and ornithine with Δ^1 -pyrroline-5-carboxylate (P5C) as the obligatory intermediate (Figure 1). The endogenous synthesis of proline, though, is not utilized to provide substrate for protein synthesis, as proline and the other nonessential amino acids are mostly acquired from dietary protein. The biosynthesis of proline through such pathways has additional metabolic functions that exploit proline's structural distinctiveness. The lack of a primary amino group makes proline immune to decarboxylation and transamination catalysis by the generic amino acid enzymes. Instead, a specific set of enzymes completely independent from those associated with other amino acids are responsible for the manipulations of proline.

P5C, in tautomeric equilibrium with glutamic- γ -semialdehyde (GSA), is the obligate substrate for proline biosynthesis, and is reduced to proline by the cytosolic NAD(P)H-dependent enzyme P5C reductase (P5CR). Proline oxidase [POX; also known as proline dehydrogenase (PRODH)] tightly bound to the mitochondrial inner membrane catalyzes the degradation of proline back to P5C. The oxidation of proline by POX to yield P5C and the conversion of P5C into proline by P5CR constitutes a proline-P5C cycle that involves two subcellular compartments, mitochondrion and cytosol. This proline-P5C cycle plays an important role in the regulation of gene expression, purine biosynthesis, cellular redox state, apoptosis, and cell proliferation (Phang et al., 2001, Hu et al., the issue). Importantly, P5C is also found in circulation, indicating the presence of unidentified transport systems across the mitochondrial and plasma membrane. There are two other sources that supply P5C: ornithine in a reaction catalyzed by mitochondrial vitamin B6-dependent ornithine- δ -aminotransferase (OAT), and glutamate in a reduction reaction catalyzed by mitochondrial ATP- and NAD(P)H-dependent P5C synthase (P5CS) (Hu et al., this issue). The P5CS reaction can be reversed by mitochondrial P5C dehydrogenase (P5CDH), which converts P5C back to glutamate (Hu et al., 1996).

The special functions of proline metabolism are evident in multiple organisms. For example, proline can function as an osmoprotectant to assist in maintaining appropriate osmotic pressure in prokaryotes and plants (Verbruggen and Hermans, this issue). Proline can act as a redox shuttle in insects and even mediate parasite-induced pathophysiology in mammalian hosts (Phang et al., 2001). In mammals, proline has been observed as essential for cell mitogenic response in addition to being affiliated with p53-induced apoptosis (Hu et al., 2007). Proline biosynthesis is directly connected to the NAD(P)H/NAD(P)⁺ redox couple, suggesting the pathway's secondary role as a redox shuttle. Finally, proline synthesis, uptake, and release in synaptosomes identify it as a possible neuromodulator or neurotransmitter. Evidently, proline metabolism affects many pathways and functions distant from standard protein biosynthesis and degradation (Phang et al., 2001).

Three of the proline metabolic enzymes, POX, P5CDH, and P5CS, have been shown to be upregulated by p53, a pivotal tumor suppressor that regulates cell cycle, angiogenesis, differentiation, bioenergetics, and programmed cell death (Dan and Semenza, 1999, Vogelstein et al., 2000; Vousden and Prives, 2005). It is now well known that POX is one of the p53 downstream effectors that induces ROS- and mitochondria-mediated apoptosis, which initiates both intrinsic and extrinsic apoptotic pathways, possibly through NFAT and MEK/ERK signaling (Donald et al., 2001, Liu et al., 2006, Hu et al., 2007). Using a quantitative proteomic approach, we have shown that P5CS is upregulated by p53 in p53-induced apoptosis in DLD-1 colorectal cancer cells (Hu et al., this issue). To further investigate p53-regulated gene expression of proline metabolic enzymes at the RNA level in the same experimental conditions, in the present study we conducted Northern blot analysis.

As human health is primarily determined by common genetic components with a complex pattern of inheritance, risk of disease is therefore influenced by a combination of several different genetic, environmental, and life-style factors. The common-variant/common-disease model predicts that most risk alleles underlying complex health-related traits may be common (Reich and Lander, 2001, Goh et al., 2007). To investigate regulatory elements in the promoter and the nonsynonymous single nucleotide polymorphisms (SNPs) in the coding exons of each gene as candidate risk alleles, we conducted a thorough functional genomics analysis of the human genes encoding proline metabolic enzymes, employing recent advances in biocomputational techniques for promoter and SNP analysis.

Materials and methods

Cell Lines and culture media

DLD-1 cells (originated from a p53-null colorectal tumor) were cultured in DMEM supplemented with 10% FBS, 1X antibacterial antimycotic solution as previously described (Polyak et al., 1997). Reh cells (originated from a leukemia patient) were culture in RPMI supplemented with 10% FBS, 1X antibacterial antimycotic solution. Both cell lines were purchased from **American Type Culture Center (ATCC)**.

Isolation of Total RNA and Northern blot analysis

Samples of total RNA isolated from DLD-1 cells that were infected with p53-harboring adenovirus (AD-p53) at indicated time points were collected as previously described (Polyak et al., 1997; Liu et al., 2007). Total RNA was isolated from Reh cells using a Purescript kit (Gentra Systems, USA). Two commercially available membranes of poly (A)⁺ RNA isolated from various tissues were purchased from Clontech (Palo Alto, CA). Northern blotting, probe preparation and hybridization were conducted as previously described [Brody et al., 1989 (OAT), Dougherty et al., 1992 (P5CR), Lin et al., 1996 (POX), Hu et al., 1996 (P5CDH), and Hu et al., 1999 (P5CS)].

Functional genomic analysis

To conduct an update and thorough review of function, disease association, RNA variants, and protein isoforms of each proline metabolic enzyme, we retrieved information from the following websites and their associated links: NCBI ENTREZ databases (<http://www.ncbi.nlm.nih.gov/sites/>), such as PubMed, Gene, OMIM (Online Mendelian Inheritance of Man) and SNP; e! Emsembl (http://www.ensembl.org/Homo_sapiens/); EMBL-EBI (<http://www.ebi.ac.uk/embl/>); the Gene Index Project, the Computational Biology and Functional Genomics Laboratory, Dana-Farber Cancer Institute, Harvard University (<http://compbio.dfci.harvard.edu/tgi/>); UCSC Genome Browser (<http://genome.cse.ucsc.edu/cgi-bin/hgTracks>); and GeneCards (<http://www.genecards.org/>). To further analyze and confirm SNPs found in NCBI ENTREZ SNP site, we also utilized FASTSNP (http://fastsnp.ibms.sinica.edu.tw/pages/input_CandidateGeneSearch.jsp; Yuan et al., 2006), the Functional Single Nucleotide Polymorphism (F-SNP) database (<http://compbio.cs.queensu.ca/F-SNP/>; Lee and Shatkay, 2008) and HaploSNPer website (<http://www.bioinformatics.nl/tools/haplosnper/>; Tang et al., 2008). To analyze putative transcription factor binding sites in gene promoters, we utilized the following biocomputational programs: Alibaba 2.1 (<http://darwin.nmsu.edu/~molb470/fall2003/Projects/solorz/index.html>), Tfsitescan (<http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl>), and WWW Promoter Scan (<http://www.bimas.cit.nih.gov/molbio/proscan/>).

Results and Discussion

Upregulation of POX, P5CDH, P5CS and OAT by p53

p53, a well-characterized tumor suppressor and transactivating factor, plays a critical role in the suppression of tumorigenesis by the regulation of cell cycle progression, differentiation, angiogenesis, and the induction of programmed cell death, both apoptosis (type I) and autophagy (type II) (Vogelstein et al., 2000, Vousden and Prives, 2005, Levine et al., 2006a). Apoptosis and autophagy are essential and highly regulated physiological processes that are required for the maintenance of tissue homeostasis to eliminate unwanted or injured cells with characteristic cellular and biochemical hallmarks. In addition, p53 also regulates cell senescence/aging and metabolism (Levine et al., 2006b, Kawauchi et al., 2008). POX, also known as PIG6 (p53 induced gene 6), is one of a handful of genes whose expression is induced by p53 in p53-induced apoptosis (Polyak et al., 1997). Subsequently, we showed overexpression of POX together with availability of proline results in increased reactive oxygen species (ROS) production that can lead to mitochondria- and caspase 8-mediated apoptosis. These perturbations may play a key role in oncogenesis in certain types of cell and tissue. (Maxwell and Rivera, 2003, Liu et al., 2006, Hu et al., 2007). Another one of the enzymes P5CDH has also been shown to be upregulated by p53 (Yoon et al., 2004). Furthermore, we recently showed that P5CS is upregulated by p53 in p53-induced apoptosis in DLD-1 colorectal cancer cells (Hu et al., this issue). Of note, however, none of these findings were obtained from the same group, the same cell line and the same treatment conditions. In the present study, in the same experiments, we showed that human POX, OAT, P5CDH and P5CS were upregulated in DLD-1 by p53 at the RNA level 24 hours, 3 hours, 9 hours, and 3 hours after p53 overexpression, respectively (Figure 2). Therefore, our results in p53-induced upregulation of POX, P5CDH and P5CS are consistent with previous findings. Human OAT is a nuclear-encoded mitochondrial matrix enzyme which catalyzes the reversible interconversion of ornithine and α -ketoglutarate to GSA/P5C and glutamate. Inherited deficiency of OAT results in ornithine accumulation and a characteristic chorioretinal degeneration, gyrate atrophy of the choroid and retina. Human OAT has been well-studied biochemically and genetically (Valle and Simell, 2001). Upregulation of OAT by p53 is a novel observation that deserves further investigation.

Human P5CR1 and P5CR2

The isozymes of human P5C reductase (P5CR; EC1.5.1.2) catalyze ATP- and NAD(P)H-dependent reduction conversion of P5C to proline, the first committed step in de novo biosynthesis of proline. For this conversion to take place P5C must leave the mitochondria because P5CR isozymes are localized to the cytoplasm and/or loosely associated with the cytosolic side of the outer mitochondrial membrane (Hu et al, unpublished data). This mechanism is important for the transfer of oxidizing potential across the cell (Phang, 1985). There are two P5CR isozymes, P5CR1 and P5CR2, encoded by two structural genes (Dougherty et al., 1992). (Dougherty and colleagues 1992) cloned human cDNAs encoding P5CR1 in the pre-EST database era by functional complementation in a proline auxotrophy strain “*pro3*” of yeast *S. cerevisiae* which lacked P5CR. They then utilized low-stringency library screening to identify a second P5CR cDNA P5CR2, and showed that both P5CR1 and P5CR2 cDNA were able to confer proline prototrophy to the P5CR-deficient yeast strain.

(Merrill and colleagues 1989) studied the properties of human erythrocyte P5CR and concluded that in addition to the traditional role of catalyzing the obligatory and final unidirectional step in proline biosynthesis, isozymes of P5CR may play a physiologic role in the generation of NADP⁺ in some types of cell including erythrocytes, and is subject to negative feedback inhibition by its own product proline and NAD(P)⁺. The normal abundance of P5CR in the cell is maintained relatively low due to its high turnover. Interestingly, a recent study by

(Krishnan and colleagues 2008) showed overexpression of P5CS and P5CR1 resulted in 2-fold higher proline content, significantly lowered ROS levels, and increased cell survival relative to control cells. Another study showed that P5CR1 activity was increased in pulmonary and colorectal tumors (Meng 2006a). Mammalian P5CR2 shows similar enzyme activity and 81% amino acid sequence identity with P5CR1. Importantly, using quantitative proteomics analysis, (Moron and colleagues 2007) recently showed that P5CR2 is identifiable in mouse hippocampal postsynaptic density (HPSD), an electron-dense structure, which receives and transduces synaptic information. Expression of P5CR2 was induced by morphine administration in the HPSD, implying that P5CR2 may play a role in intracellular signaling and synaptic plasticity in the brain.

In the search for human diseases associated with P5CR deficiency, we noticed that there is a proline auxotrophic human leukemic lymphoblastoid cell line Reh, a human B cell precursor leukemia cell line established from the peripheral blood of a 15-year-old North African girl with acute lymphoblastic leukemia in 1973. It has been shown that Reh cells are deficient of NADH-dependent P5CR activity, but express normal NADPH-dependent P5CR activity (Lorans and Phang, 1981), suggesting that there are multiple isozymes of P5CR. Molecular cloning of two types of cDNA and the identification of two structural genes encoding P5CR1 and P5CR2 explain this phenomenon. To examine tissue expression of human P5CR2, we conducted Northern blot analysis of poly(A)⁺ RNA from multiple human tissues and showed a single predominant P5CR2 transcript of about 1.85 kb. Testis, ovary, small intestine, leukocyte and colon had the highest expression followed by spleen, prostate, thymus, placenta, pancreas and liver (Figure 3A). We also conducted Northern blot analysis on RNA isolated from Reh cells and control CCL-114 cells and showed that P5CR2 was highly expressed in Reh cells. In contrast, expression of P5CR1 was lost in Reh cells (Figure 3B). This result together with earlier biochemical studies of P5CR activities in these cells indicate P5CR2 utilizes NADPH while P5CR1 utilizes NADH. We speculate that various combinations of expression of P5CR1 and P5CR2 account for the tissue variations in the biochemical characteristics of P5CR activity.

The structural gene of human P5CR1 [also known as PYCR1; proliferation-inducing protein 45 (PIG45); GeneID: 5831] is localized on chromosome 17q25.3, possesses 4 exons and spans ~5 kb. There are two types of P5CR1 transcript variant, 1 and 2, generated by alternative splicing, differing in the last 90 bps at the 3' end. Variant 1 encodes the long 319-aa isoform P5CR1.1, whereas Variant 2 encodes the short 316-aa isoform P5CR1.2. P5CR1.2 has a distinct 30-aa sequence at the C-terminus compared to P5CR1.1 (Table 1). The SNP databases reported two nonsynonymous SNPs in the coding exons of P5CR1 which result in 2 different codon changes, K289R and G297R (Table 2). In addition, extended promoter analysis showed putative binding sites for p53, c-fos, c-jun and c-myc in the promoter region of the P5CR1 gene (Table 3).

The structural gene of human P5CR2 (also known as PYCR2; GeneID: 29920) is localized on chromosome 1q42.12, comprises 7 exons and spans ~4.4 kb. We identified two nonsynonymous SNPs in P5CR2 coding exons that result in one codon alteration, M1I, and one frameshift mutation, 253insT. Both SNPs/alleles presumably would cause loss-of-function consequence. We also found that there are several putative binding sites for p53, c-fos, c-jun and c-myc were observed in the promoter region of P5CR2 gene through promoter analysis (Table 3). Based on the roles of transcription factors p53, c-fos, c-jun and c-myc, we hypothesize that expression of P5CR1 and P5CR2 can be regulated by apoptosis, cell growth/proliferation, and bioenergetics. In fact, P5CR1 is also known as proliferation-inducing protein 45 (PIG45).

Importantly, purification and crystal structure of human P5CR1 have been reported (Meng 2006a, b). The 2.8 Angstroms (Å) resolution structure of the P5CR1 apo enzyme and its 3.1 Å resolution ternary complex with NAD(P)H and substrate-analog demonstrated that human P5CR1 possesses a decameric architecture with five homodimer subunits and ten catalytic sites arranged around a peripheral circular groove.

Human POX/PRODH and OH-POX

Proline oxidase (POX) also known as proline dehydrogenase (PRODH; EC 1.5.99.8) is a mitochondrial inner-membrane enzyme which catalyzes the first step in the proline degradation pathway, converting proline to P5C by use of flavin adenine dinucleotide as a cofactor. POX/PRODH uses proline to generate ATP and ROS which can be used for either survival or apoptosis. It is induced by p53 under various stresses and initiates apoptosis by both mitochondrial (intrinsic) and death receptor (extrinsic) pathways. In addition, POX/PRODH is induced by PPAR γ , and is upregulated under nutrient stress through the mTOR pathway (Phang et al., this issue). Previously using biochemical assays, enzymatic activity of POX was found primarily in the liver, kidney, and brain (Phang et al., 2001).

Human PRODH1 (GeneID: 5625) structural gene is localized on chromosome 22q11.21, comprises 15 exons and spans 23.77 kb, and encodes a 600-amino acid POX/PRODH protein (Lin et al., 1996, Bender et al., 2005). Expression of PRODH1 is inducible by p53 and known as PIG6 (P53 Induced Gene 6; Polyak et al., 1997). Missense mutations on the PRODH1 gene are linked to multiple disease states, type I hyperprolinemia (HPI), non-specified hyperprolinemia, velocardiofacial syndrome/DiGeorge syndrome (VCFS/DGS), CATCH 22 syndrome and schizophrenia (Jacquet et al., 2002, Liu et al., 2002, Bender et al., 2005). While disease states result from inactive POX, overexpression of POX has been shown to cause both increased ROS generation and apoptosis that is proline dependent (Hu et al. 2007). The PRODH1 gene is a hot spot for mutations: sixteen reported missense mutations in the POX gene have been found. These mutations cause mild to severe effects in POX activity: four SNPs/alleles (R185Q, L289M, A455S, and A472T) result in mild (<30%), six (Q19P, A167V, R185W, D462N, V427M, and R431H) in moderate (30%–70%), and five (P406L, L441P, R453C, T466M, and Q521E) in severe (>70%) reduction in POX activity. Three of the mutations, V427M, L441P, and R453C, linked to severe reduction in POX activity, were also associated with or found in schizophrenia. Interestingly, one SNP/allele (Q521R) increases POX activity (Bender et al., 2005). Through an extensive SNP database analysis, we found 7 novel nonsynonymous SNPs in the coding exons of the human PRODH1 gene resulting in one premature termination, R185stop, and 6 different codon alterations, P30S, L79F, T275N, T349A, T357P, and A367P (Table 2). All 7 of these SNPs/alleles presumably would cause drastic consequences. Sequence analysis on the human PRODH1 promoter region showed that there is one putative p53 binding sequence. This further confirms previous findings that POX is inducible by p53. Subsequent promoter analysis showed other putative sites for c-jun, c-myc and glucocorticoid responsive element (GRE) (Table 3). This is consistent with the previous observations that expression of PRODH/POX can be regulated by glucocorticoid (Kowaloff et al., 1977 and 1978).

It is worth noting that hydroxyproline (OH-Pro) and proline are metabolized by distinct pathways. The first steps in the degradation of OH-Pro is catalyzed by mitochondrial OH-Pro oxidase (OH-POX; EC unknown). This reaction is important in catabolism of OH-Pro found primarily as an oligopeptide in body fluids and posttranslationally produced by hydroxylation of proline residues in the nascent collagen polypeptide chains. Free hydroxyproline is derived from endogenous collagen turnover and from breakdown of dietary collagen (Hu et al., 2001; Phang et al., 2001). Deficiency of OH-POX causes hyperhydroxyprolinemia, an autosomal recessive disease characterized by at least 10-fold accumulation of plasma OH-Pro

(140–500 μ M). This metabolic disorder was initially described in association with mental retardation, but subsequent identification in clinically normal individuals has led to the supposition that it is benign (Kim et al., 1997). The human OH-POX structural gene (PRODH2; GeneID, 58510) is localized on chromosome 19q13.1, and has 12 exons distributed over 17 kb of genomic DNA. The OH-POX cDNA has a 1608 bp ORF encoding a protein of 536 residues with a predicted molecular mass of 58 kDa (Table 1, Hu et al., 2001). Cooper and colleagues recently showed that OH-POX is inducible by p53 (Cooper et al., 2008). We conducted a thorough SNP database analysis and found 4 nonsynonymous SNPs in the coding exons of the human OH-POX which result in 4 different codon alterations, P91R, R177Q, A210D, and R525Q (Table 2). All 4 of these SNPs/alleles could presumably cause drastic consequences in OH-POX activity.

Human P5CDH

Human P5C dehydrogenase (P5CDH; EC 1.5.1.12) is a mitochondrial matrix NAD^+ -dependent dehydrogenase which converts P5C to glutamate. It has been purified from the human liver and found to be a “high K_m ” aldehyde dehydrogenase (ALDH) with GSA as a primary substrate. The purified enzyme from rat liver was found to exhibit activity with other aldehydes, therefore, P5CDH belongs to the ALDH family and is known as ALDH4A1 (ALDH, family 4, subfamily A, member 1) (Vasiliou et al., 1999; Table 1). (Hu and colleagues 1996) cloned the full-length cDNAs encoding human P5CDH which encodes a 563-amino acid protein with a putative 24-amino acid N-terminal mitochondrial targeting sequence. Deficiency of P5CDH is associated with type II hyperprolinemia (HPII), an autosomal recessive disorder characterized by accumulation of P5C and proline (Valle et al., 1979). Although HPII has been considered a benign disorder, further research into this metabolic disorder indicates that HPII causes clinical manifestations. The study of 13 cases in 1 Irish traveler’s pedigree strongly supports a causal relationship between HPII and neurological manifestations. This large pedigree of Irish travelers, a distinct nomadic group within the Irish population with many individuals affected by HPII (Table 2; Flynn et al., 1989; Geraghty et al., 1998). Approximately 70% of affected members of this pedigree had childhood febrile seizures but mental handicap was not a feature. Four HPII probands have been studied to date with identification of four mutant alleles. Pathological mutations of human P5CDH gene, two with frameshift and 1 with a missense, have been found in 3 unrelated probands with HPII. A frameshift mutation consisted of insertion of a T following nucleotide 1563 and causing a frameshift at codon G521 in the P5CDH gene in homozygous state in affected members. In one segment of the pedigree the father and 6 of 7 children were all homozygous for the mutant allele, the mother was heterozygous, and 1 unaffected child was heterozygous. In addition, we found compound heterozygosity for mutations in the P5CDH gene: a C-to-T transition at nucleotide 1055 resulting in a S352L missense mutation, and a C-to-G transversion at nucleotide 1050 resulting in a synonymous mutation, A350A, in cis with the S352L mutation in a HPII patient. Finally, we also found homozygosity for a 1-bp deletion (G) at nucleotide 21 of the P5CDH cDNA, resulting in a frameshift mutation in codon 7 for alanine. This same mutation was also present in compound heterozygous state with the S352L mutation in another family (Table 2; Geraghty et al., 1998).

Human P5CDH structural gene (ALDH4A1; GeneID: 8659) is localized on chromosome 1p36, possesses 16 exons and spans ~50 kb. Two P5CDH transcript variants can be generated by alternative splicing. The two human P5CDH cDNAs identified differed only by retention of a 1-kb intron in the 3’ untranslated sequence. The longer transcript is more common in most tissues. Less than 5% of reported cDNAs have the presence of an intron in the 3’ untranslated sequence in mammalian transcripts. However, both mature transcripts encode only one, 563-aa P5CDH polypeptide (Table 1; Hu et al., 1996). Expression of P5CDH is inducible by p53 in response to DNA damage caused by adriamycin treatment. It is speculated that here p53

might play a protective role against cell damage induced by generation of intracellular ROS, in part, through transcriptional activation of P5CDH (Yoon et al., 2004). Our Northern blot analysis confirmed that expression of human P5CDH was indeed inducible by p53 9 hours after p53 overexpression (Figure 2).

Aside from the 4 pathological SNPs/alleles found in HP11 patients, five new nonsynonymous SNPs in P5CDH coding exons resulting in five different codon changes, E227K, G366R, F388L, V407I and T473A, have been reported in the SNP databases (Table 2). Some of these SNPs/alleles may cause functional consequences and deserve further functional analysis. In addition, one p53, three c-jun and one c-myc putative binding sites in the P5CDH promoter region were observed through computational promoter analysis (Table 3).

Human P5C synthase (P5CS)

P5CS is a bifunctional ATP- and NAD(P)H-dependent mitochondrial enzyme that catalyzes the coupled phosphorylation and reduction-conversion of glutamate to P5C, a pivotal step in the biosynthesis of proline, ornithine and arginine. We previously reported cloning and characterization of two P5CS transcript variants generated by exon sliding that encode two protein isoforms differ only by a 2 amino acid-insert at the N-terminus of the γ -glutamyl kinase active site. The short form (P5CS.short) is highly expressed in the gut and is inhibited by ornithine. In contrast, the long form (P5CS.long) is expressed ubiquitously and is insensitive to ornithine (Hu et al., 1999). Deficiency of P5CS in 2 children born from a consanguineous marriage showed phenotypic features included hyperammonemia, hypoprolinemia, hypocitrullinemia, and hypoorithinemia with joint hyperlaxity, skin hyperelasticity, cataract, and mental retardation. Both patients were homozygous for a G-to-A transition at position 251 of the P5CS gene, resulting in an arg84-to-gln (R84Q) substitution. The R84Q mutation alters a conserved residue in the P5CS γ -GK domain and dramatically reduces the activity of both P5CS isoforms when expressed in mammalian cells. Additionally, R84Q appears to destabilize the long isoform (Baumgartner et al., 2000). We recently reported regulation of P5CS expression by p53 and growth hormones in cultured human cell lines (Hu et al., this issue). Our Northern blot analysis confirmed that expression of P5CS was indeed upregulated by p53 3 hours after p53 overproduction (Figure 2).

The human P5CS structural gene, also known as ALDH18A1 (aldehyde dehydrogenase family member 18A1; GeneID: 5832; Vasiliou et al., 1999), is located on chromosome 10q24.3 and spans 15 kb. Aside from the known pathological R84Q SNP/allele that caused P5CS deficiency in two sibs, we identified 9 new nonsynonymous SNPs in P5CS coding exons resulting in two different frameshift mutations, 78insG and 1092insG, and 7 different codon changes, R38K, S264P, T2P7I, Q363Ter, S370Y and V592I (Table 2). Obviously, three SNPs/alleles, two frameshift mutations, 78insG and 1092insG, and one premature termination, Q363Ter, of P5CS would presumably cause loss-of-function phenotype, whereas other SNPs may also cause functional consequences. A recent study by (Tadros and colleagues 2007) demonstrated that P5CS was downregulated with age and with hearing loss in the mouse auditory midbrain. They hypothesized that since P5CS plays a role in converting glutamate to proline, P5CS deficiency in old age may lead to both glutamate increases and proline deficiencies in the auditory midbrain, and may play a role in the subsequent inducement of glutamate toxicity through the loss of proline neuroprotective effects. It would be of importance to investigate whether or not these newly identified SNPs of P5CS play any role in age-related hearing loss.

Finally, putative binding sites for p53, c-jun, c-myc and GRE were observed in the P5CS promoter region through promoter analysis. In addition, there is a putative P53 binding site in the first intron (intron 1) of the P5CS gene (Table 3). Taken together, the identification of p53-binding consensus sequence in the cis-regulatory sequences of P5CS, and the induction of P5CS by p53 at the RNA and proteomic levels (Hu et al., this issue) confirm that P5CS is a

bona fide p53 downstream target. In addition, a GRE was found in the P5CS promoter region, suggesting as previously studied, that expression of P5CS is regulated by glucocorticoids (Wu et al., 2000). In fact, our recent results showed that expression of human P5CS is regulated by growth hormones, such as glucocorticoids and estrogen (Hu et al., this issue).

Future perspectives

Proline metabolism is purpose-driven, tightly regulated, and compartmentalized in mammalian cells. It involves two other amino acids, glutamate and ornithine, and five enzymatic activities, P5CR, POX, P5CDH, P5CS and OAT. With the exception of OAT, which catalyzes a reversible reaction, the other 4 enzymes are unidirectional. This tri-amino-acid system also links with three other essential metabolic systems, namely the TCA cycle, urea cycle, and pentose phosphate pathway. Abnormalities in proline metabolism are relevant in several diseases: six known monogenic inborn errors involving metabolism and/or transport of proline and its immediate metabolites have been described. In addition, impaired proline metabolism has been implicated as a susceptibility factor for schizophrenia, a complex neuropsychiatric disorder with a frequency of ~1% around the world. Our future investigations will focus on functions and roles of the SNPs in proline metabolic enzymes and their association with disease states, role of intracellular accumulation of GSA/P5C in cellular cytotoxicity, and link between POX and P5CR in cancers and P5CS in age-related hearing loss.

Abbreviations

GRE, Glucocorticoid responsive element
 OAT, Ornithine- δ -aminotransferase
 OH-POX, Hydroxyproline oxidase
 OMIM, Online Mendelian Inheritance in Man
 P5C, Δ^1 -pyrroline-5-carboxylate
 P5CDH, P5C dehydrogenase
 P5CS, P5C synthase
 P5CR, P5C reductase
 POX, Proline oxidase
 PRODH, Proline dehydrogenase
 SNP, Single nucleotide polymorphism

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REFERENCES

- Baumgartner MR, Hu CAA, Almashanu S, Steel G, Obie C, Aral B, Rabier D, Kamoun P, Saudubray J-M, Valle D. Hyperammonemia with reduced ornithine, citrulline, arginine and proline: a new inborn error caused by a mutation in the gene encoding delta-1-pyrroline-5-carboxylate synthase. *Hum Mol Gen* 2000;9:2853–2858. [PubMed: 11092761]
- Bender H, Almashanu S, Steel GCA, Lin W-W, Willis A, Pulver A, Valle D. Functional Consequences of PRODH Missense Mutations. *Am. J. Hum. Genet* 2005;76:000–000.
- Böck A, Forchhammer K, Heider J, Leinfelder W, Sawers G, Veprek B, Zinoni F. Selenocysteine: the 21st amino acid. *Mol. Microbiol* 1991;5:515–520. [PubMed: 1828528]

- Brody LC, Mitchell GA, Obie C, Michaud J, Steel G, Fontaine G, Robert MF, Sipila I, Kaiser-Kupfer M, Valle D. Ornithine delta aminotransferase mutations in gyrate atrophy. Allelic heterogeneity and functional consequences. *J Biol Chem* 1992;267:3302–3307. [PubMed: 1737786]
- Cooper SK, Pandhare J, Donald SP, Phang JM. A novel function for hydroxyproline oxidase in apoptosis through generation of reactive oxygen species. *J Biol Chem* 2008;283:10485–10492. [PubMed: 18287100]
- Dang CV, Semenza GL. Oncogenic alterations of metabolism. *Trends Biochem Sci* 1999;24:68–72. [PubMed: 10098401]
- Donald SP, Sun XY, Hu CA, Yu J, Mei JM, Valle D, Phang JM. Proline oxidase, encoded by p53-induced gene-6, catalyzes the generation of proline-dependent reactive oxygen species. *Cancer Res* 2001;61:1810–1815. [PubMed: 11280728]
- Dougherty KM, Brandriss MC, Valle D. Cloning human pyrroline-5-carboxylate reductase cDNA by complementation in *Saccharomyces cerevisiae*. *J Biol Chem* 1992;267:871–875. [PubMed: 1730675]
- Flynn MP, Martin MC, Moore PT, Stafford JA, Fleming GA, Phang JM. Type II hyperprolinaemia in a pedigree of Irish Travellers (nomads). *Arch Dis Child* 1989;64:1699–1707. [PubMed: 2624476]
- Geraghty MT, Vaughn D, Nicholson AJ, Lin WW, Jimenez-Sanchez G, Obie C, Flynn MP, Valle D, Hu CAA. Mutations in the delta-1-pyrroline 5-carboxylase dehydrogenase gene cause type II hyperprolinemia. *Hum Molec Genet* 1998;7:1411–1415. [PubMed: 9700195]
- Goh K-I, Cusick ME, Valle D, Childs B, Vida M, Barabasi A-L. The human disease network. *Proc Natl Acad Sci* 2007;104:8685–8690. [PubMed: 17502601]
- Hao B, Gong W, Ferguson TK, James CM, Krzycki JA, Chan MK. A new UAG-encoded residue in the structure of a methanogen methyltransferase. *Science* 2002;296:1462–1466. [PubMed: 12029132]
- Hu CAA, Lin WW, Valle D. Cloning, characterization and expression of cDNAs encoding human Δ^1 -pyrroline-5-carboxylate dehydrogenase. *J Biol Chem* 1996;271:9795–9800. [PubMed: 8621661]
- Hu CAA, Lin WW, Obie C, Valle D. Molecular enzymology of mammalian delta1-pyrroline-5-carboxylate synthase. Alternative splice donor utilization generates isoforms with different sensitivity to ornithine inhibition. *J Biol Chem* 1999;274:6754–6762. [PubMed: 10037775]
- Hu CAA, Lin WW, Steel G, Levy H, Valle D. Identification of the gene encoding hydroxyproline oxidase and delineation of mutations responsible for hyperhydroxyprolinemia. *Am J Hum Genet* 2001;69:S1708.
- Hu CAA, Donald SP, Yu J, Lin WW, Liu Z, Steel G, Valle D, Phang JM. Overexpression of proline oxidase induces proline-dependent and mitochondria-mediated apoptosis. *Mol Cell Biochem* 2007;295:85–92. [PubMed: 16874462]
- Jacquet H, Raux G, Thibaut F, Hecketsweiler B, Houy E, Demilly C, Haouzir S, Allio G, Fouldrin G, Drouin V, Bou J, Petit M, Campion D, Frébourg T. PRODH mutations and hyperprolinemia in a subset of schizophrenic patients. *Hum Mol Genet* 2002;11:2243–2249. [PubMed: 12217952]
- Kawauchi K, Araki K, Tobiume K, Tanaka N. p53 regulates glucose metabolism through an IKK-NF-kappaB pathway and inhibits cell transformation. *Nat Cell Biol*. 2008[Epub April 6, 2008]
- Kim SZ, Varvogli L, Waisbren SE, Levy HL. Hydroxyprolinemia: comparison of a patient and her unaffected twin sister. *J Pediatr* 1997;130:437–441. [PubMed: 9063421]
- Krishnan N, Dickman MB, Becker DF. Proline modulates the intracellular redox environment and protects mammalian cells against oxidative stress. *Free Radic Biol Med* 2008;44:671–681. [PubMed: 18036351]
- Kowaloff EM, Granger AS, Phang JM. Glucocorticoid control of hepatic proline oxidase. *Metabolism* 1977;26:893–901. [PubMed: 875734]
- Kowaloff EM, Phang JM, Granger AS, Downing SJ. Glucocorticoid induction of praline oxidase in LLC-RK1 cells. *J Cell Physiol* 1978;97:153–159. [PubMed: 701384]
- Lee PH, Shatky H. F-SNP: computationally predicted functional SNPs for disease association studies. *Nucleic Acids Res* 2008;36:D820–D824. [PubMed: 17986460]
- Levine AJ, Feng Z, Mak TW, You H, Jin S. Coordination and communication between the p53 and IGF-1-AKT-TOR signal transduction pathways. *Genes Dev* 2006a;20:267–275. [PubMed: 16452501]
- Levine AJ, Hu W, Feng Z. The P53 pathway: what questions remain to be explored? *Cell Death Differ* 2006b;13:1027–1036. [PubMed: 16557269]

- Lin WW, Hu CAA, Valle D. Molecular cloning of cDNAs encoding human and mouse proline oxidase, the enzyme-deficient in type I hyperprolinemia and Pro/Re mice. *Am J Hum Genet* 1996;59:A269.
- Liu Y, Borchert GL, Surazynski A, Hu CAA, Phang JM. Proline oxidase activates both intrinsic and extrinsic pathways for apoptosis: the role of ROS/superoxides, NFAT and MEK/ERK signaling. *Oncogene* 2006;25:5640–5647. [PubMed: 16619034]
- Liu Z, Wan G, Heaphy C, Bisoffi M, Griffith JK, Hu CAA. A novel loss-of-function mutation in TP53 in an endometrial cancer cell line and uterine papillary serous carcinoma model. *Mol Cell Biochem* 2007;297:179–187. [PubMed: 17119852]
- Lorans G, Phang JM. Proline synthesis and redox regulation: differential functions of pyrroline-5-carboxylate reductase in human lymphoblastoid cell lines. *Biochem Biophys Res Commun* 1981;101:1018–1025. [PubMed: 6946770]
- Maxwell SA, Rivera A. Proline oxidase induces apoptosis in tumor cells, and its expression is frequently absent or reduced in renal carcinomas. *J Biol Chem* 2003;278:9784–9789. [PubMed: 12514185]
- Meng Z, Lou Z, Liu Z, Hui D, Bartlam M, Rao Z. Purification, characterization, and crystallization of human pyrroline-5-carboxylate reductase. *Protein Exp Purif* 2006a;49:83–87.
- Meng Z, Lou Z, Liu Z, Hui D, Bartlam M, Rao Z. Crystal Structure of Human Pyrroline-5-carboxylate Reductase. *J Mol Biol* 2006b;359:1364–1377. [PubMed: 16730026]
- Merrill MJ, Yeh GC, Phang JM. Purified human erythrocyte pyrroline-5-carboxylate reductase. Preferential oxidation of NADPH. *J Biol Chem* 1989;264:9352–9358. [PubMed: 2722838]
- Morón JA, Abul-Husn NS, Rozenfeld R, Dolios G, Wang R, Devi LA. Morphine administration alters the profile of hippocampal postsynaptic density-associated proteins: a proteomics study focusing on endocytic proteins. *Mol Cell Proteomics* 2007;6:29–42. [PubMed: 17028301]
- Phang JM. The regulatory functions of proline and pyrroline-5-carboxylic acid. *Curr Topics Cell Regul* 1985;25:91–132.
- Phang, JM.; Hu, CAA.; Valle, D. Disorders of proline and hydroxyproline metabolism. In: Scriver, CR.; Beaudet, AL.; Sly, WS.; Valle, D., editors. *Metabolic and Molecular Basis of Inherited Disease*. New York: McGraw Hill Press; 2001. p. 1821–1838.
- Polyak K, Xia Y, Zweier JL, Kinzler KW, Vogelstein B. A model for p53-induced apoptosis. *Nature* 1997;389:300–305. [PubMed: 9305847]
- Reeds PJ. Dispensable and indispensable amino acids for humans. *J Nutr* 2000;130:1835S–1840S. [PubMed: 10867060]
- Reich DE, Lander ES. On the allelic spectrum of human disease. *Trends Genet* 2001;17:502–510. [PubMed: 11525833]
- Srinivasan G, James CM, Krzycki JA. Pyrrolysine encoded by UAG in Archaea: charging of a UAG-decoding specialized tRNA. *Science* 2002;296:1459–1462. [PubMed: 12029131]
- Tadros SF, D'Souza M, Zettel ML, Zhu X, Waxmonsky NC, Frisina RD. Glutamate-related gene expression changes with age in the mouse auditory midbrain. *Brain Res* 2007;1127:1–9. [PubMed: 17113045]
- Tang J, Leunissen JA, Voorrips RE, van der Linden CG, Vosman B. HaploSNPer: a web-based allele and SNP detection tool. *BMC Genet* 2008;9:23. [PubMed: 18307806]
- Valle D, Goodman SI, Harris SC, Phang JM. Genetic evidence for a common enzyme catalyzing the 2nd step in the degradation of proline and hydroxyproline. *J Clin Invest* 1979;64:1365–1370. [PubMed: 500817]
- Valle, D.; Simell, O. Hyperornithinemia. In: Scriver, CR.; Beaudet, AL.; Sly, WS.; Valle, D., editors. *Metabolic and Molecular Basis of Inherited Disease*. New York: McGraw Hill Press; 2001. p. 1857–1895.
- Vasilou V, Bairoch A, Tipton KF, Nebert DW. Eukaryotic aldehyde dehydrogenase (ALDH) genes: human polymorphisms, and recommended nomenclature based on divergent evolution and chromosomal mapping. *Pharmacogenetics* 1999;9:421–434. [PubMed: 10780262]
- Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature* 2000;408:307–310. [PubMed: 11099028]
- Vousden KH, Prives C. P53 and prognosis: new insights and further complexity. *Cell* 2005;120:7–10. [PubMed: 15652475]

- Wu G, Knabe DA. Arginine synthesis in enterocytes of neonatal pigs. *Comp Physiol* 1995;269:R621–R629.
- Wu G, Meininger CJ, Kelly K, Watford M, Morris SM Jr. A cortisol surge mediates the enhanced expression of pig intestinal pyrroline-5-carboxylate synthesis during weaning. *J Nutri* 2000;130:1914–1919.
- Yoon K-A, Nakamura Y, Arakama H. Identification of ALDH4 as a p53-inducible gene and its protective role in cellular stresses. *J Hum Genet* 2004;49:134–140. [PubMed: 14986171]
- Yuan HY, Chiou JJ, Tseng WH, Tseng WH, Liu CH, Liu CK, Lin YJ, Wang HH, Yao A, Chen YT, Hsu. FASTSNP: an always up-to-date and extendable service for SNP function analysis and prioritization. *Nucleic Acids Res* 2006;34:W635–W641. [PubMed: 16845089]

Fig 1.
Metabolic pathways concerning proline in mammalian cells (see text for details).

Fig 2.

Regulation of expression of human genes encoding P5CS, OAT, POX and P5CDH by p53 (see text for details). P21 was used as a positive control p53 inducible gene. β -actin was used as a loading control.

Fig 3.

Northern blot analysis of human P5CR2 expression in various human tissues and Reh cells. a Expression of P5CR2 investigated in 16 different tissues (see text for details). b P5CR2 is highly expressed in Reh cells. In contrast, expression of P5CR1 is lost in Reh cells. CCL-114 is a control B lymphoblast cell line. β-actin was used as a loading control.

Table 1
Human proline metabolic enzymes: from genes to mRNA variants to protein isozymes

Enzyme	Gene Name	Gene ID	OMIM#	Map Location	# of Exons	P53 Inducible	mRNA, bp	ORF, bp	Isozyme	# Amino Acids
P5CR1 [*]	PYCR1; P5CR1	5831	N/A	17q25.3	7	N/A	2059	957	P5CR1.1	319
P5CR2	PYCR2; P5CR2	29920	N/A	1q42.12	5	N/A	1768	948	P5CR1.2	316
POX	PRODHL; PIG6; POX	5625	606810	22q11.21	15	Yes	1708	960	P5CR.2	320
			239500				2400	1,800	POX	600
OH-POX	PRODH2	58510	237000	19q13.1	12	Yes	1,667	1,608	OH-POX	536
P5CDH	ALDH4A1; P5CDH	8659	606811	1p36	16	Yes	3601	1,689	P5CDH	563
			238510				2147			
P5CS	ALDH18A1; PYCS; P5CS	5832	138250	10q24.3	18	Yes	3470	2,385	P5CS.long	795
							3464	2,379	P5CS.short	793

^{*} P5CR1 is also known as proliferation inducing Gene 45 (PIG45)

Table 2

Nonsynonymous SNPs in human genes encoding proline metabolic enzymes

Gene	Exon	Non-synonymous change in the coding sequence ^{*,a}	Codon change	Significance
P5CR1	7	T866C	K289R	Presumably drastic
	7	G889A	G297R	Presumably drastic
P5CR2	1	G3A	M1I	Presumably nonfunctional
	3	253ins	Frame shift	Presumably nonfunctional
PRODH1/POX	2	G56T	Q19P	Presumably subtle
	2	G88A	P30S	Presumably drastic
	2	C237G	L79F	Presumably drastic
	4	C500T	A167V	Presumably subtle
	5	A553G	R185W	Presumably subtle
	5	C554T	R185Q	Presumably subtle
	5	G554T	R185stop	Presumably nonfunctional
	7	G824T	T275N	Presumably drastic
	8	C866T	L289M	Pathologic ^b
	10	T1045C	T349A	Presumably drastic
	10	G1071C	T357P	Presumably drastic
	10	C1099G	A367P	Presumably drastic
	11	G1217A	P406L	Pathologic ^b
	12	G1278A	D426N	Pathologic ^c
	12	C1279T	V427M	Pathologic ^{b,c}
	12	C1292T	R431H	Pathologic ^c
	12	A1322G	L441P	Pathologic ^{b,c}
	12	G1357A	R453C	Pathologic ^{b,c}
	12	C1363A	A455S	Pathologic ^c
	12	G1397A	T466M	Presumably drastic
	12	C1414T	A472T	Presumably subtle
	14	C1561G	Q521E	Pathologic ^c
	14	A1562G	Q521R	Presumably subtle
PRODH2/OH-POX	3	G272C	P91R	Presumably drastic
	4	C530T	R117Q	Presumably drastic
	5	G629T	A210D	Presumably drastic
	12	C1574T	R525Q	Presumably drastic
ALDH4A1/P5CDH	1	G21del1bp	A7fs(-1)	Pathologic ^d
	1	C47T	P16L	Pathologic ^d
	8	G829A	E277K	Presumably drastic
	10	C1055T	S352L	Pathologic ^d
	10	G1096A	G366R	Presumably drastic
	11	T1162C	F388L	Presumably drastic

Gene	Exon	Non-synonymous change in the coding sequence ^{*,a}	Codon change	Significance
ALDH18A1/P5CS	13	C1408A	V470I	Presumably subtle
	13	A1417G	T473A	Presumably drastic
	16	1563insT	G521fs(+1)	Pathologic ^d
	2	78insG	Frame shift	Presumably nonfunctional
	3	G113A	R38K	Presumably drastic
	3	G251A	R84Q	Pathologic ^e
	7	T790C	S264P	Presumably drastic
	8	A889C	T297P	Presumably drastic
	8	C890T	T297I	Presumably drastic
	10	C1087T	Q363Ter	Presumably nonfunctional
	10	1092insG	Frame shift	Presumably nonfunctional
	10	C1109A	S370Y	Presumably drastic
	14	G1774A	V592I	Presumably subtle

* sense strand coding sequence, +1 represents the A of the first ATG

^a newly identified SNPs in this study are in bold

^b Bender et al., 2005

^c Liu et al., 2002

^d Geraghty et al., 1998

^e Baumgartner et al., 2000

Table 3
Summary of the putative transcription factor binding sites in the promoter region of the human genes encoding proline metabolic enzymes

GENE	TRANSCRIPTION FACTORS (# of hits)				
	p53	c-fos	c-jun	c-myc	GRE
P5CR1	2	1	3	2	0
P5CR2	3	2	2	4	0
PRODHL/POX	1	0	2	2	1
ALDH4A1/P5CDH	1	0	3	1	0
ALDH18A1/P5CS/PYCS	2*	0	1	2	1

*. There are two putative p53 binding sites, one in the promoter and one in the intron 1 of this gene; GRE stands for glucocorticoid responsive element