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ORIGINAL ARTICLE

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

Chien-an A. Hu · D. Bart Williams · Siqin Zhaorigetu · Shadi Khalil · Guanghua Wan · David Valle

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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 four 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

Abbreviations

GRE	Glucocorticoid responsive element
OAT	Ornithine-D-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

C. A. Hu (✉) · D. Bart Williams · S. Zhaorigetu · S. Khalil · G. Wan
Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, Albuquerque, NM 87131, USA
e-mail: ahu@salud.unm.edu

D. Valle
Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Introduction

With the addition of two new members, selenocysteine (Böck et al. 1991) and pyrolysine (Hao et al. 2002; Srinivasan et al. 2002), there are now 22 known natural, genetically encoded, proteingenic amino acids in living organisms. Proline, 1 of the 22 proteingenic amino acids, is traditionally categorized as 1 of the nonessential amino acids in mammals because there is a specific set of enzymes designated to synthesize proline from its precursors in mammalian 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 substrates 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 (Fig. 1). The endogenous synthesis of proline, though, is not mainly 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). Significantly, P5C is also found in circulation, indicating the presence of unidentified transport systems across the mitochondrial and plasma membranes. 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).

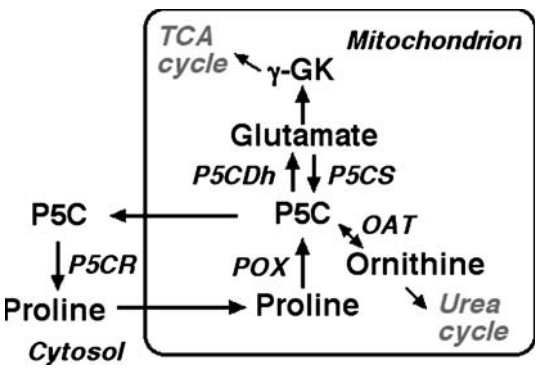

Fig. 1 Metabolic pathways concerning proline in mammalian cells (see text for details)

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 pathways and functions distant from standard protein biosynthesis and degradation (Phang et al. 2001).

Recently, 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 (Dang 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. It 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). Furthermore, using a quantitative proteomic approach, we recently showed that P5CS long 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, 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 cultured in RPMI supplemented with 10% FBS, 1X antibacterial antimycotic solution. Both cell lines were purchased from 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 collected as previously described [Brody et al. 1992 (OAT); Dougherty et al. 1992 (P5CR); Lin et al. 1996 (POX); Hu et al. 1996 (P5CDH); 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 by eliminating 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, overexpression of POX together with availability of proline results in increased reactive oxygen species (ROS) production that can lead to mitochondria- and caspase eight-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 experiment, we showed that human POX, OAT, P5CDH and P5CS were upregulated in DLD-1 by p53 24, 3, 9, and 3 h after p53 overexpression, respectively (Fig. 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

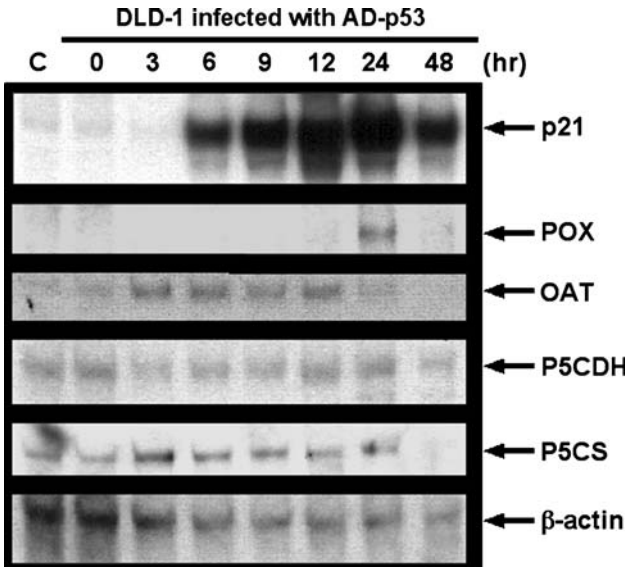

Fig. 2 Regulation of expression of human genes encoding P5CS, OAT, POX and P5CDH by p53 (see text for details). P21 was used as a positive control p53 inducible gene. β-actin was used as a loading control

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 et al. (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 et al. (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 et al. (2008) showed overexpression of P5CS and P5CR1 resulted in twofold higher proline content, significantly lowered ROS levels, and increased cell survival relative to control cells. Another study showed that P5CR1 activity is increased in pulmonary and colorectal tumors (Meng et al. 2006a). Mammalian P5CR2 shows similar enzyme activity and 81% amino acid sequence identity with P5CR1. Importantly, using quantitative proteomics analysis, Morón et al. (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.

While searching for human diseases associated with P5CR deficiency, we noticed 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 which 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 (Fig. 3a). We also conducted Northern blot analysis on RNA isolated from Reh cells and control CCL-114 cells which showed that P5CR2 was highly expressed. In contrast, expression of P5CR1 was lost in Reh cells (Fig. 3b). This result together with earlier biochemical studies of P5CR activities in these cells indicate that 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);

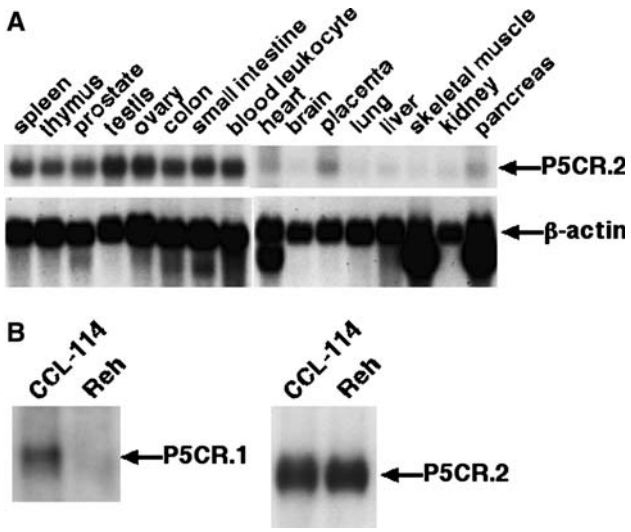

Fig. 3 Northern blot analysis of human P5CR2 expression in various human tissues and Reh cells. **a** Expression of P5CR2 in 16 different tissues have been investigated (see text for details). **b** P5CR2 is highly expressed in Reh cells. In contrast, expression of P5CR1 is lost in Reh cells. CCL-114 is a control B lymphoblast cell line. *β*-actin was used as a loading control

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 two 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, and 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 et al. 2006a, b). The 2.8 Angstroms (A) resolution structure of the P5CR1 apo enzyme and its 3.1A 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

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

Enzyme	Gene name	Gene ID	OMIM no.	Map location	No. of exons	P53 inducible	mRNA (bp)	ORF (bp)	Isozyme	No. amino acids
P5CR1[a]	PYCR1; P5CR1	5831	N/A	17q25.3	7	N/A	2,059	957	P5CR1.1	319
							1,768	948	P5CR1.2	316
P5CR2	PYCR2; P5CR2	29920	N/A	1q42.12	5	N/A	1,708	960	P5CR.2	320
POX	PRODH1, PIG6, POX	5625	606810	22q11.21	15	Yes	2,400	1,800	POX	600
			239500							
OH-POX	PRODH2	58510	237000	19q13.1	12	Yes	1,667	1,608	OH-POX	536
P5CDH	ALDH4A1; P5CDH	8659	606811	1p36	16	Yes	3,601	1,689	P5CDH	563
			238510				2,147			
P5CS	ALDH18A1; PYCS; P5CS	5832	138250	10q24.3	18	Yes	3,470	2,385	P5CS.long	795
							3,464	2,379	P5CS.short	793

[a] 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 sequnce[a,b]	Codon change[b]	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[c]
	10	**T1045C**	**T349A**	Presumably drastic
	10	**G1071C**	**T357P**	Presumably drastic
	10	**C1099G**	**A367P**	Presumably drastic
	11	G1217A	P406L	Pathologic[c]
	12	G1278A	D426N	Pathologic[d]
	12	C1279T	V427M	Pathologic[c,d]
	12	C1292T	R431H	Pathologic[d]
	12	A1322G	L441P	Pathologic[c,d]
	12	G1357A	R453C	Pathologic[c,d]
	12	C1363A	A455S	Pathologic[d]
	12	G1397A	T466M	Presumably drastic
	12	C1414T	A472T	Presumably subtle
	14	C1561G	Q521E	Pathologic[d]
	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	G21del1 bp	A7fs (−1)	Pathologic[e]
	1	C47T	P16L	Pathologic[e]
	8	**G829A**	**E277K**	Presumably drastic
	10	C1055T	S352L	Pathologic[e]
	10	**G1096A**	**G366R**	Presumably drastic
	11	**T1162C**	**F388L**	Presumably drastic
	13	**C1408A**	**V470I**	Presumably subtle
	13	**A1417G**	**T473A**	Presumably drastic
	16	1563insT	G521fs(+1)	Pathologic[e]
ALDH18A1/ P5CS	2	**78insG**	**Frame shift**	Presumably nonfunctional
	3	**G113A**	**R38 K**	Presumably drastic
	3	G251A	R84Q	Pathologic[f]
	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

[a] Sense strand coding sequence, +1 represents the A of the first ATG

[b] Newly identified SNPs in this study are in bold

[c] Bender et al. (2005)

[d] Liu et al. (2002)

[e] Geraghty et al. (1998)

[f] Baumgartner et al. (2000)

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 PPARγ, and is upregulated by nutrient stress through the mTOR pathway (Phang et al. this issue). Previously, by using biochemical assays, enzymatic activity of POX was found primarily in the liver, kidney, and brain (Phang et al. 2001), as well as small intestine (Wu 1997) and placenta (Wu et al. 2005).

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 (no. of hits)				
	p53	c-fos	c-jun	c-myc	GRE
P5CR1	2	1	3	2	0
P5CR2	3	2	2	4	0
PRODH1/POX	1	0	2	2	1
ALDH4A1/P5CDH	1	0	3	1	0
ALDH18A1/P5CS/PYCS	2[a]	0	1	2	1

GRE glucocorticoid responsive element

[a] There are two putative p53-binding sites, one in the promoter and one in the intron 1 of this gene

Human PRODH1 (GeneID: 5625) structural gene is localized on chromosome 22q11.21, comprises 15 exons, span: 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, MIM 239500), non-specified hyperprolinemia, velocardiofacial syndrome/DiGeorge symdrome (VCFS/DGS), CATCH 22 syndrome and schizophrenia (Jacquet et al. 2002; Liu et al. 2002a, b; 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: 16 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; Willis et al. this issue). Through an extensive SNP database analysis, we found seven novel nonsynonymous SNPs in the coding exons of the human PRODH1 gene resulting in one premature termination, R185stop, and six different condon alterations, P30S, L79F, T275 N, T349A, T357P, and A367P (Table 2). All seven 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, 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 post-translationally 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 tenfold accumulation of plasma OH-Pro (140–500 μM). 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 1,608 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 four nonsynonymous SNPs in the coding exons of the human OH-POX, which result in four different codon alterations, P91R, R177Q, A210D, and R525Q (Table 2). All four of these could SNPs/alleles 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 km" 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 et al. (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, MIM

239510), 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 one 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 one with a missense, have been found in three unrelated probands with HPII. A frameshift mutation consisted of insertion of a T following nucleotide 1563 and causing a frameshift at codon 521 in the P5CDH gene in homozygous state in affected members. In one segment of the pedigree the father and six of seven children were all homozygous for the mutant allele, the mother was heterozygous, and one 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 h after p53 overexpression (Fig. 2).

Aside from the four pathological SNPs/alleles found in HPII 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 two 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 two children born from a consanguineous marriage showed phenotypic features including hyperammonemia, hypoprolinemia, hypocitrullinemia, and hypoornithinemia 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 h after p53 overproduction (Fig. 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 nine new nonsynonymous SNPs in P5CS-coding exons resulting in two different frameshift mutations, 78insG and 1092insG, and seven 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 et al. (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 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 indeed 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 four 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 $\sim 1\%$ 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.

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