Analysis of DPYD Gene Using Bioinformatics Tools

DPYD GENİNİN BİYOİNFORMATİK ARAÇLARLA ANALİZİ

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Abstract

Objective: The *DPYD* gene, which encodes for the dihydropyrimidine dehydrogenase (DPD), is responsible for the pyrimidine catabolic pathway, and degrades more than 80% of the administered chemotherapeutic drug 5-fluorouracil (5-FU). This study aimed to investigate *In silico* the structure of the *DPYD* gene and its products.

Material and Methods: We investigated the homology, conserved domain, promoter and expression profiles of human *DPYD* gene in various species using bioinformatics tools, such as NCBI blast, EBI ClustalW, DigiNorthern, Mega3, and Genomatix programs.

Results: Our results revealed that DPD proteins were conserved among all organisms investigated. They had three conserved domains (GltD, DHPD_FMN, and COG1146), some of which had full and truncated sub-domains. We noted that the human DHPD_FMN and COG1146 domains were more conserved among the investigated species than the human GltD domain. With the multiple alignment strategy, protein and GltD domain sequences of *Xenopus tropicalis (X. tropicalis)* was predicted to have a truncation. The comparative screening of the promoters demonstrated that DPYD genes did not seem to have any common conserved transcription factor binding sites.

Conclusion: This study demonstrated that the DPD molecules in various species, except *Danio rerio (D. rerio)*, were well conserved throughout evolution. Comparative screening of the promoter sequences of the human *DPYD* gene and its homologues found in the NCBI database revealed that there was no any common transcription factor binding sites.

Key Words: Dihydrouracil dehydrogenase (NADP), genomics, evolution, promoter regions (Genetics), gene expression

Özet

Amaç: *DPYD* geni, kemoterapik bir ilaç olan 5-fluorouracil (5-FU)'in %80'den fazlasını parçalayan ve pirimidin katabolik yolunda sınırlayıcı olan dihidropirimidin dehidrogenaz (DPD)'ı kodlar. Bu çalışma, çeşitli türlerdeki *DPYD* genini biyoinformatik olarak araştırmayı amaçlamaktadır.

Gereç ve Yöntemler: NCBI Blast, EBI ClustalW, DigiNorthern, Mega3 ve Genomatix gibi biyoinformatik araçları kullanarak insan *DPYD* geninin homolojisini, korunan alanlarını, promotor ve ekspresyon profillerini çeşitli türlerde inceledik.

Bulgular: Sonuçlarımız, DPD protein dizisinin incelenen tüm organizmalar arasında korunduğunu gösterdi. İnsan DPD proteini, 3 korunan bölge ailesi (GltD, DHPD_FMN ve COG1146) vardır ve bunlardan bazılarının tam ve kesikli alt korunan bölgeye sahip oldukları belirlendi. İncelenen türler arasında, insan DHPD_FMN ve COG1146 bölgeleri, insan GltD bölgesinden daha fazla korunduğu görülmüştür. Çoklu sıraya dizme stratejisi ile *Xenopus tropicalis (X. tropicalis)*'in tüm protein ve GltD korunan dizilerinin eksik olduğu görülmüştür. Promotorların karşılaştırmalı olarak taranması ile *DPYD* geninin genel olarak herhangi bir korunan traskripsiyon faktör bağlama alanlarının olmadığı belirlendi.

Sonuçlar: Bu çalışma, *Danio rerio (D. rerio)* dışındaki çeşitli türlerde DPD proteinlerinin evrimsel süreç boyunca korunduğunu gösterdi. NCBI bilgi bankasında bulunan insan *DPYD* geni ve onun homologlarının karşılaştırmalı taranması, herhangi bir ortak transkripsiyon faktör bağlama bölgesinin olmadığını gösterdi.

Anahtar Kelimeler: Dihidrourasil dehidrogenaz (NADP), genomik, evrim, promotor bölgeleri, gen ekspresyonu

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The human DPYD gene, also known as (DPI) gene, is a single copy gene on chromosome 1p22; consisting of 23 exons, at least 950 kb in length. It encodes a DHPD of approximately 111-kDa.[1-4] DHPD protein shows cytoplasmic localization and is more sensitive to 5-FU used in the treatment of cancer.[5,6]

The cDNAs coding for human, pig and bovine DHPDs have been isolated and sequenced.[7] Mammalian DHPD appears to be relatively conserved throughout evolution, since a comparison of the deduced amino acid sequences of bovine DHPD

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with that of pig and human DHPD shows 93% and 92% homology, respectively.[8,9]

Comparative analysis of the uracil and NADPH binding sites in mammals and invertebrates demonstrated 100% amino acid identity between mammals and *Drosophila melanogaster*. Caenorhabditis elegans demonstrated 89% and 88% identity in these domains (Uracil and NADPH binding sites), respectively.[10]

DHPD enzyme activity shows high variation in the analyzed population and is correlated with DPYD mRNA expression.[11,12] The expression levels of DPYD gene may be useful indicators of 5-FU activity in lung cancer.[13,14] DHPD activity was significantly higher in breast cancer tissues than in adjacent normal breast tissue.[15] Reports indicated that there were two regulatory elements in the DPYD promoter.[16]

In this study, we aimed to analyze the DPYD genes in different species *In silico*, specifically, their GltD and DHPD_FMN domains, the transcription factor binding sites (TFBs) on their promoters, the tissue expression profile, homology level and phylogenetic tree among vertebrate DPYD genes, using bioinformatics tools.

Material and Methods

Homology Search

The search for homologous protein sequence to human DHPD was carried out using the BLASTp program at NCBI (http://www.ncbi.nlm.nih.gov) using human DHPD amino acid sequence (GI: 4503373) as query against the SwissProt protein databases.[17,18] Full protein, NADPH-dependent glutamate synthase beta chain and related oxidoreductases (GltD), dihydropyrimidine dehydrogenase (DHPD) FMN-binding domain (DHPD_FMN), and Ferredoxin (COG1146) sequences of human and other species were downloaded and then aligned using the Clustal W program at the EBI site (http://www.ebi.ac.uk).[19]

Promoter Analysis

We used Genomatix software (http://www.genomatix.de) for analysis of DPYD gene promoters in various species. These nucleotide sequences were downloaded and aligned using the ClustalW program. Then common TFBs were searched for with the Dialign TF program in the Genomatix software for all DPYD promoters present in the database.

Evolutionary Analysis

We used amino acid sequences of GltD, DHPD_FMN, and COG1146 domains to construct phylogenetic trees using the neighbor-joining method (NJ) with Jones-Taylor-Thomton (JTT) distances. NJ searches were conducted by using MEGA3 and 500 bootstrap replicates were assessed for the reliability of internal branches; sites with gaps were ignored in this analysis.[20]

In Silico Expression Analysis

The DigiNorthern database was used to analyze the expression of DPYD mRNAs based on EST data.[21] The DigiNorthern collects all ESTs for a query gene and categorizes those ESTs based on the types of tissues and their histological status. Pair wise comparisons of relative values were performed with the Fisher's exact test using SPSS 11.0 for Windows.

Results

Homology Search

BLASTp results revealed that DHPD molecule was conserved in various species (Table 1). The homology search indicated that the DHPD sequences of *Pan troglodytes (P. troglodytes)* (99%), and *Macaca mulatta* (*M. mulatta)* (98%), had the highest homology to that of human. In contrast, the one with the lowest homology to human DHPD protein was that of *D. rerio* (76%*)* (Table 1 and Figure 1).

ClustalW alignment elucidated the presence of well conserved three domains: GltD, DHPD_FMN, and COG1146 domains, which were well conserved especially in some localized areas (Figure 1). "RTTYGGVSG", "IRPIALRAV", "IARALP", "FPILATGGIDSAESGLQFLH" "GASVLQVCSA", and "QNQDFT" motifs in the DHPD_FMN domains and "EMCINCGKCYMTCNDSGYQAI", "ETHL" and "CTGCTLCLSVCPI" in the COG1146 were well conserved in all investigated

Table 1. BLASTp results of vertebrate DHPD molecules and their homology.

Species	Common name	Acession no	Length (amino acid)	% Identity with human DPD
Homo sapiens	Human	NP000101	1025	100
Pan troglodytes	Chimpanzee	XP513583	1219	99
Macaca mulatta	Rhesus monkey	XP001106007	1025	98
Sus scrofa	Pig	NP999209	1025	92
Bos taurus	Cow	NP776466	1025	92
Mus musculus	Mouse	NP740748	1025	89
Rattus norvegicus	Rat	NP112289	1025	89
Gallus gallus	Chicken	XP426639	1178	82
Xenopus tropicalis	Frog	CAJ82653	275	78
Danio rerio	Zebrafish	NP998058	1022	76

species (Figure 1). These regions are most probably functionally important and any mutations in them are deleterious, as implicated by their evolutionary conservation.

Multiple alignment results of human DHPD and its homologous revealed that this molecule was well conserved in the investigated species. The finding that DHPD molecules of *P. troglodytes* and *Gallus gallus (G. gallus)* were longer than those of other species (an extra 394 and 360 amino acids at the N-termini, respectively) was the main difference. The remaining molecules of other species displayed a very similar pattern.

We determined three conserved domain families: GltD, DHPD_FMN, and COG1146 domains. GltD is NADPH-dependent glutamate synthase beta chain and related oxidoreductases, while DHPD_FMN is a DHPD FMN-binding domain, which catalyzes the first step in pyrimidine degradation and it contains two FAD, two FMN, and eight [4Fe_4S] clusters and COG1146 is ferredoxin domain, which regulates energy production and conversion. In addition, we observed some sub-domains within these domains. For example GltD domain harbored overlapping Pyr-redox (pyridine nucleotide disulphide oxidoreductase), TrxB (thioredoxin reductase), Lpd (pyruvate/2-oxoglutarate dehydrogenase complex), and Ndh (NADH dehydrogenase, FAD-containing subunit) domains, while DHPD_FMN harbored DHOD_DHPD_FMN (Dihydroorotate dehydrogenase), oxidored_FMN (NADPH-dependent flavin oxidoreductase dehydrogenase (DHPD) FMN binding domain), PyrD (Dihydroorotate dehydrogenase), and DHO_dh (Dihydroorotate dehydrogenase).

Furthermore, we also detected some partial subdomains in the three conserved domain families, which were truncated at the N- and C-termini. They were ignored in analysis due to the inconsistency and truncation.

Promoter Analysis

Analyzing the promoters present in the database of Genomatix software, we failed to detect any common TFBs in the DPYD promoters of *H. sapiens, M. mulatta, Rattus norvegicus (R. norvegicus), Mus musculus (M. musculus)* and *G. gallus*. However, we observed that the similarity (value 1.000) and the percentage of identical nucleic acids (in short sequence segments) was 69% between the DPYD promoters of *Homo sapiens (H. sapiens)* and *M. mulatta* for each pairwise alignment.

Evolutionary Analysis

From the phylogenetic trees constructed by MEGA3, we found that the GltD, DHPD_FMN, and COG1146 domains of DPYD genes were conserved among all organisms investigated. We showed that the GltD, DHPD_FMN, and COG 1146 domains of *H. sapiens, P. troglodytes* and

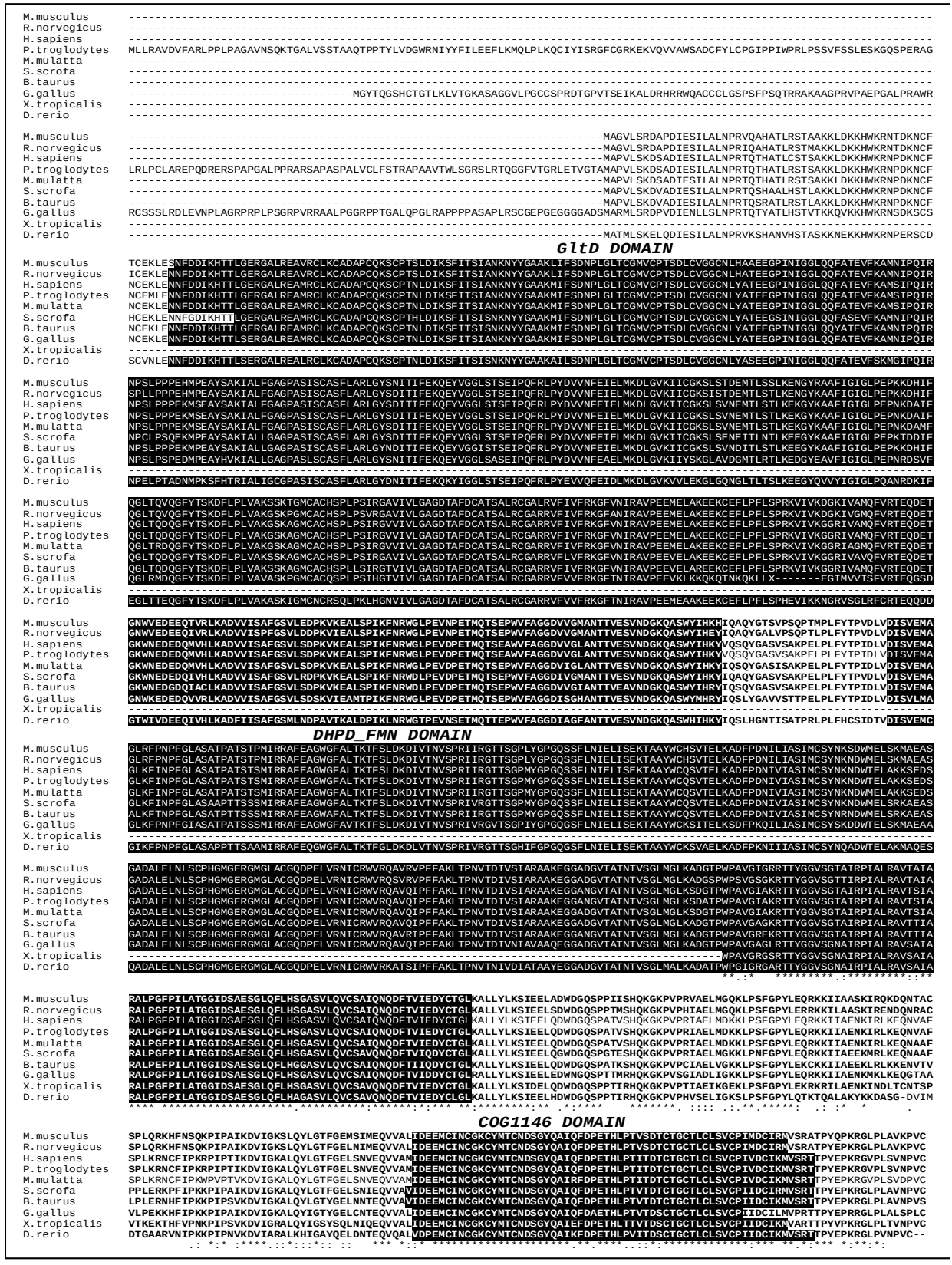

Figure 1. Multiple alignments of vertebrate DHPD proteins. The GltD, DHPD_FMN, and COG1146 domains are highlighted with black background. The conserved amino acid residues are shown by an asterisk and amino acids with similar properties are shown by a semi-colon under the alignment.

M. mulatta were more closely grouped (scale length 0.02, 0.01, and 0.001, respectively) (Figure 2A, B, and C). In contrast, we observed that these domains of *D. rerio* showed the lowest similarity to those of humans. When we constructed the phylogenetic tree, we ignored domain sequences of GltD of *X. tropicalis* due to its high diversity caused by possible truncation (Figure 1).

In Silico Expression Analysis

The distribution of human DPYD ESTs in the cDNA library database (all ESTs) was analyzed using the DigiNorthern program. In columns 2 and 3 of Table 2, relative values of DPYD ESTs in the cDNA libraries from normal and tumor tissues respectively are shown both as absolute numbers as well as normalized values per 10^6 cDNAs. Its normal as well as cancerous tissue expression profile was compared and the significance in its expression pattern was assessed with the Fisher's exact test (p value 0.05) (Table 2). Human DPYD gene was expressed at low or high levels in some tissues, but was not expressed in others. The tissue distribution and differential expression pattern in normal and cancerous human tissues displayed somehow different values. The expression of DPYD gene in some normal human tissues, such as bone marrow, cartilage, cervix, colon, gastrointestinal tract, genitourinary, lymphoreticular, and ovary, was not detected. In contrast, its expression seems to be lost in some cancerous tissues, such as adipose, bone, brain, eye, lung, mammary gland, muscle, nervous, pancreatic islet, placenta, and prostate. Compared to normal tissues, its expression was significantly decreased (p=0.003) in cancerous bone marrow tissues (Table 2).

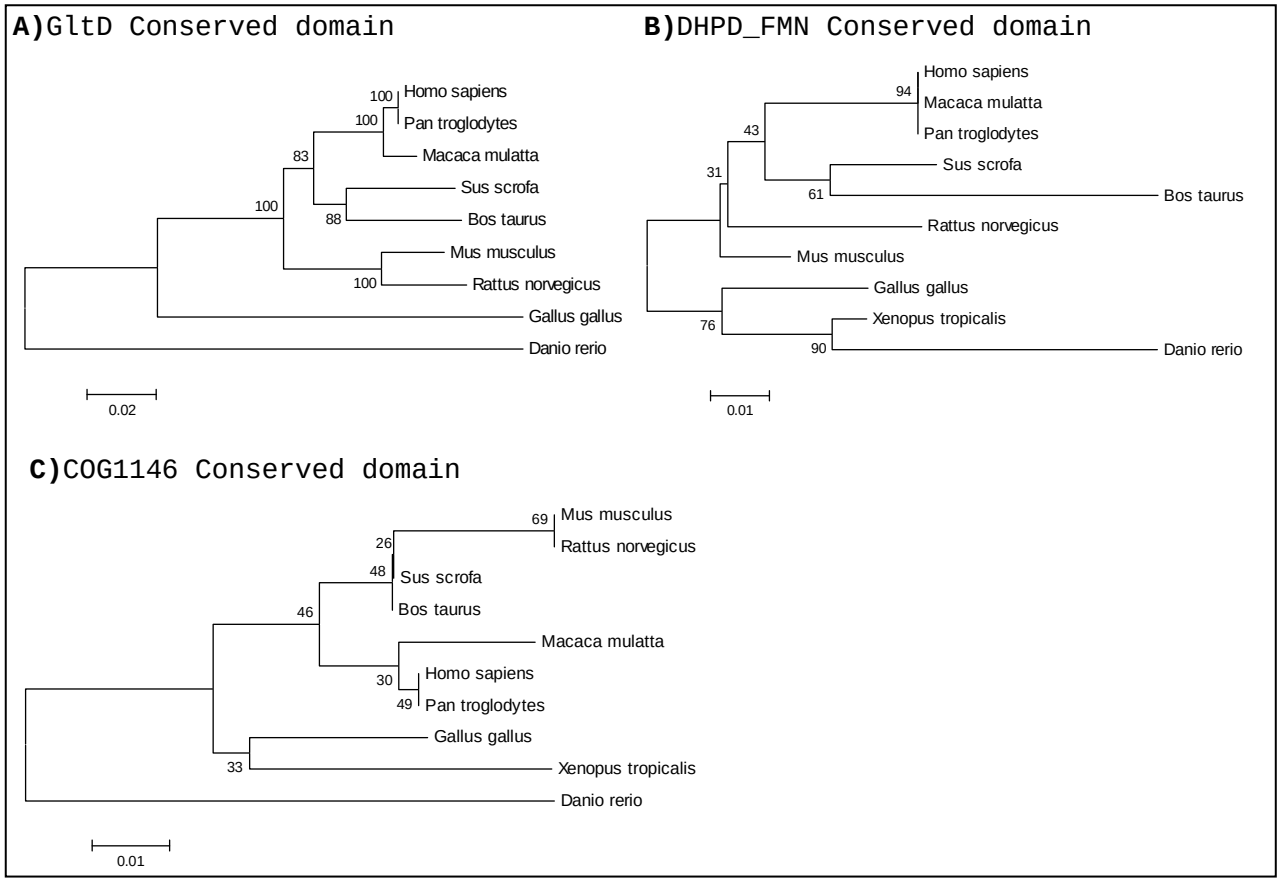

Figure 2. Phylogenetic tree of GltD (**A**), DHPD_FMN (**B**), and COG1146 (**C**) domains of vertebrate DHPD. Phylogenetic trees were constructed using the MEGA3 program. Species names are indicated on the figure. Branch lengths indicate evolutionary relationship.

Absolute numbers and the relative values as normalized per 10^6 cDNAs (in parenthesis) in normal and cancerous tissues are shown; p-values are for comparison of relative values of DPYD ESTs in normal versus tumor tissues, using the Fisher's exact test (last column). The data for tissues with significant or suggestive significant higher or lower value of DPYD ESTs in the tumor and normal tissues are shown in bold.

Discussion

Human DHPD is a pyrimidine catabolic enzyme (1025 amino acids) with a calculated molecular weight of 111 kDa. It catalyzes the initial and rate-limiting step in the pathway of uracil and thymidine catabolism and also in the pathway leading to the formation of 5'-fluorodeoxyuridine triphosphate (5'-FdUTP), then to 5'-fluorodeoxyuridine monophosphate (5'-FdUMP) inside the cell.

Table 2. Comparison of relative values of human DPYD cDNA in cDNA library database from specific normal and tumor tissues.

	Number of ESTs		
Tissue/organ type	**Normal**	**Cancer**	**p-value**
Adipose	1/11923 (84)	0/1740 (0)	1.000
Bone	**3/7929 (38)**	**0/45730 (0)**	**0.003**
Bone marrow	0/21187 (0)	1/29586 (34)	1.000
Brain	2/257019 (8)	0/201219 (0)	0.507
Cartilage	0/13369 (0)	2/39893 (50)	1.000
Cervix	0/1157 (0)	1/44671 (22)	1.000
Colon	0/28085 (0)	1/220946 (5)	1.000
Eye	1/85966 (12)	0/49827 (0)	1.000
Gastrointestinal tract	0/796 (0)	2/14690 (136)	1.000
Genitourinary	0/1687 (0)	1/39698 (25)	1.000
Head and neck	1/55508 (18)	4/107902 (37)	0.668
Kidney	3/74917 (40)	1/96375 (10)	0.325
Liver	8/73021 (110)	3/81780 (37)	0.130
Lung	3/129822 (23)	0/207630 (0)	0.057
Lymph node	2/97096 (21)	5/54341 (92)	0.106
Lymphoreticular	0/15679 (0)	1/56791 (18)	1.000
Mammary gland	1/71315 (14)	0/124006 (0)	0.365
Muscle	4/90941 (44)	0/45799 (0)	0.308
Nervous	1/15506 (64)	0/63270 (0)	0.197
Ovary	0/11587 (0)	4/109344 (37)	1.000
Pancreatic islet	3/95891 (31)	0/0 (0)	1.000
Peripheral nervous system	2/30154 (66)	0/1220 (0)	1.000
Placenta	5/248276 (13)	0/43818 (0)	1.000
Pooled tissue	6/373366 (16)	2/55060 (36)	0.275
Prostate	3/82545 (36)	0/81283 (0)	0.250
Skin	1/49729 (20)	2/137037 (15)	1.000
Stem cell	1/184378 (5)	0/0 (0)	1.000
Stomach	0/26066 (0)	1/140405 (7)	1.000
Testis	3/122158 (25)	1/44649 (22)	1.000
Thymus	0/5359 (0)	0/201 (0)	1.000
Uncharacterized tissue	1/88784 (11)	1/105216 (10)	1.000
Uterus	0/36080 (0)	11/163186 (67)	0.233
Vascular	4/31425 (127)	0/0 (0)	1.000
Whole body	1/73648 (14)	0/0 (0)	1.000
Total No. of ESTs Found	**60/2512369 (24)**	**44/2407313 (18)**	**0.202**

The latter metabolite inhibits the enzyme thymidylate synthetase, which is essential for the synthesis of thymidine, one of the four nucleotides from which DNA is constructed.[6]

Our BLASTp results indicate that DHPD molecule is present in various species of vertebrates and these molecules have 76-99% conservation degree in the total amino acid sequences (Table 1). The human DHPD molecule has the highest homology to those of *P. troglodytes* (99%) and lowest homology to that of *D. rerio* (76%). So, these results indicate that the DPYD gene has been evolutionary well conserved (Table 1). Recently, it was shown that the bovine DPYD was homologous to those of human (92%) and pig (93%). In this way, mammalian DPYD gene appears to be relatively conserved throughout evolution.[9] We also examined the phylogenetic trees of GltD, DPHD_FMN, and COG1146 domains of DHPD in different species using MEGA3 program. We observed that human GltD, DPHD_FMN, and COG1146 domain proteins showed the closest homology to those of *P. troglodytes* and *M. mulatta*, but not to those of *D. rerio* (Figure 2A, B, and C). These domains are very important for their functions. We did not phylogenetically analyze the GltD domain of *X. tropicalis* due to its incomplete sequence. Recently, a comparison study on the uracil and NADPH binding sites in mammals and invertebrates demonstrated 100% amino acid identity between the DHPDs of mammals and *Drosophila melanogaster*. This comparative analysis identified conserved regions which may be critical for enzyme structure and/or function.[10] When we compared the conserved DHPD_FMN and COG1146 domains among different species, we found that "RTTYG-GVSG", "IRPIALRAV", "IARALP", "FPILAT-GGIDSAESGLQ FLH", "GASVLQVCSA", and "QNQDFT" motifs in the DHPD_FMN domains and "EMCINCGKCYMTC-NDSGYQAI", "ETHL" and "CTGCTLCLSVCPI" in the COG1146 were well conserved in all investigated species (Figure 1). "EMCINCGKCY MTCNDSGYQAI" and "CTGCTLCLSVCPI" motifs in COG1146 (ferredoxin) coordinated 3rd and 4th Fe-S motifs. It was shown that the Iron-sulphurs (Fe-S) was focused to Cysteine (C) on the motifs "CINCGKCYMTCN" and "CTGCTL CLSVCP".[10] "EMCINCGKCYMT CNDSGYQAI" and "CTGCT LCLSVCPI" motifs in COG1146 included the "CINCGKCYMTCN" and "CTGCTL CLSVCP". The importance of these and other motif sequences need to be experimentally defined.

The expression of human DPYD gene in different tissues was analyzed using the DigiNorthern program (Table 2). Its expression patterns in normal and cancer tissues displayed somehow different values in human. In some tissues, such as bone marrow, cartilage, cervix, ovary, thymus, and uterus, its expressions seemed to be at a very low level or not at all. In cancerous adipose, mammary gland and placenta its expression was not observed while it was decreased in cancerous bone tissue. In contrast, its expression was significantly decreased in cancerous muscle tissues ($p=0.003$) (Table 2). We consider that the change in DPYD expression pattern in different tissues may have a role in pathogenesis of some sporadic cancers. The availability of comprehensive data generated by high-throughput functional genomic approaches, mainly expressed sequence tag (EST) and serial analysis of gene expression (SAGE), provides the feasibility to study gene expression analysis.[22] Miyoshi et al demonstrated that DHPD protein expression levels significantly correlated with histological grade and tumor size. They analyzed mRNA expression of DPYD gene by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and found that DPYD expression level in poorly differentiated prostate cancer was significantly lower than that in prostate cancer with more favorable well or moderately differentiated-histopathology.[14] Likewise, the expression levels of DPYD gene were suggested to be a useful indicator of 5-FU activity in lung cancer.[13] DHPD protein was reported to directly catabolize 5-FU to adenine. This suggests that this DHPD activity may provide a target for 5-FU use in cancer therapy.[9]

We used Dialign TF program in Genomatix software for predicting TFBs (transcriptional elements) of all orthologous DPYD promoters present

in the database. Dialign TF results revealed that DPYD orthologous promoters had no common conserved transcriptional elements. The conservation of transcriptional elements in promoter sequences may provide further evidence in support of functional conservation.[23-25] However, the element in the promoters or their vicinity may be more mobile than the genes themselves. Our results indicate that the binding sites of different transcription factors might have located on different parts of the promoter or promoter vicinity in various species.

These findings provide the foundation for future investigations of the molecular mechanisms underlying the heterogeneity of DHPD activity in humans. Basic bioinformatics techniques are powerful tools in terms of leading to the discoveries and analysis of novel genes[26]. Recently, we identified and further characterized two novel genes using bioinformatics tools.[27,28] Even though the results from bioinformatics studies are very helpful in directing and designing the experiments, they need to be supported and confirmed by further experimental data.

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