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The interaction of p53 and MDM2 genes in cancers, *in silico* studies and phylogenetic analysis

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Abstract
The normal cell cycle process is a crucial process and is generally mediated by a number of regulatory genes. One of the most important regulators is the tumor suppressor p53, which in turn is regulated by MDM2 gene. The expression of p53 and MDM2 is found to be frequently altered in many cancers and metastasis/ relapses .This is the first report to look at the evolutionary history of these genes to decipher the role of these genes in the tumorigenesis process using *in silico* methods. We also found that they showed high degree of sequence similarity across the mammalian species, indicating that these species probably share parallel cancer causing mechanisms. Their individual unrooted phylogenetic tree formed 5 clusters each; however, p53 gene was found in a large number of species whereas MDM2 was found in smaller number of species. The role of MDM2 is therefore limited and occurs in fewer species across the mammalian species. It is evident that these molecules play an important role in the cancer process, perhaps responsible for relapses and hence need to be explored further as therapeutic targets. Such studies that are based on evidence from paleontology and genetics suggest that mechanisms of cancer are embedded deeply throughout evolution. Understanding the phylogenetic evolution of these genes could help in furthering our knowledge on the mechanisms involved in cancer.

Keywords: p53; MDM2; genes; cancer; *in silico* studies; phylogeny; cell cycle regulation.

Introduction

Neoplasia is a heterogeneous disease affecting mankind at all stages of life. To better understand the etiology of the disease, it is necessary to understand the molecular mechanisms that lead to the production of cancer cells. Abnormalities in the cell cycle process have largely been implicated in the prognosis of cancer. The cell cycle process plays a crucial role in the development of an organism as it controls cellular growth, differentiation and proliferation processes. Any change in the cell cycle would successively alter these processes and thus lead to cancer. The normal cell cycle process thus becomes a crucial process and is generally mediated by a number of regulatory genes. These genes include cyclins, cyclin dependent kinases and their inhibitors, many tumor suppressor genes, which ensure the proper progression of the cell cycle from one phase to another and also ensure the appropriate termination of the cell cycle process. In a previous study, Khan and Jamil (2008) carried out cancer metasignature gene analysis and reported that genes involved in tumorigenesis are involved in multiple pathways essential for cell survival and hence contribute to sustained proliferation of cancer cells.

The expression of p53 tumor suppressor gene and other genes that participate in the p53 pathway are found to be frequently altered in many cancers. The p53 gene encodes a 53 kDa protein that participates in a plethora of functions. It functions as a transcription factor, regulating the expression of many genes that are essential for checkpoint response due to DNA damage and also genes that participate in apoptotic pathways (Vousden and Lane, 2007). Initial levels of p53 in cells are fairly low but gradually increase in response to genotoxic stress signals. When DNA damage has been signaled, p53 protein interrupts G1 phase of cell division and allows for repair of the damaged DNA and also activates apoptosis to remove cells that are irreparably damaged (Kastan *et al.*, 1991). Altered expression of p53 through mutations or deletions results in change in apoptosis process and might also contribute to resistance to drug therapies (Liu *et al*., 2002). Though p53 alterations are infrequent during diagnosis of ALL, in 16% to 28% of children suffering from relapse in ALL point mutations in p53 have been observed (Zhou *et al*., 1995),

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indicating a critical role of p53 in the tumorigenesis process. However, these mutations are much higher in solid tumors and other malignancies.

Since p53 has such diverse and critical functions, there exist mechanisms in the normal cells systems which regulate p53 expression so that it does not malfunction in the absence of external stress stimuli. One of the most important regulators is the MDM2 gene. The protein encoded by this gene, which is an ubiquitin ligase, binds to the N-terminal transactivation domain of the p53 molecule and thus acts as a negative regulator of p53 expression. On receiving stress stimuli, the p53-MDM2 interaction is broken allowing p53 to carry out its functional activities (Sakaguchi *et al*., 1998; Craig *et al*., 1999). MDM2 itself is as a proto-oncogenic protein wherein its overexpression leads to suppression of p53 activity thus disrupting apoptosis. Overexpression of MDM2 and its subsequent inactivation of p53 activity has been reported in several cases of childhood ALL (Marks *et al*., 1997; Gustafsson *et al*., 2001). MDM2 also participates in cell cycle regulation, proliferation and apoptosis independent of p53. MDM2 expression levels affect these processes, and are thought to contribute to its role in tumorigenesis process (Zhang and Zhang, 2005). Overexpression of MDM2 has been reported in association with early relapse and drug resistance in pediatric leukemia (Zhou *et al*., 2000).

The tumorigenesis process involves unlimited cell proliferations due to deregulation of pathways involved in cellular activities. Many of these pathways are not clearly understood in normal cells, and so information about how changes in these pathways lead to malignancies is even more difficult to decipher. Recent advances in evolutionary biology has helped provide tools so that the pathways can be studied in an evolutionary context and thus provide more information regarding the effect of alterations of genes involved in these pathways and their role in tumorigenesis. Weinberg (2007) has indicated that cancer causing genes are highly conserved and those genes that are responsible for multicellularity are most often altered leading to cancer. In previous studies, Khan and Jamil (2008) have used phylogenetic approaches and functional divergence to understand evolutionary history of MTHFR gene and its SNPs, which is reported in association with many diseases including leukemia. Their studies have implicated that SNPs in MTHFR occur in sites under functional constraint and these sites might be fixed in a particular population.

In the current study, our aim was to look at the evolutionary history of p53 and MDM2 genes through the construction of phylogenetic trees, since it may further our knowledge to predict the cell cycle process in cancers as these genes are involved in the cell division process, and further it may be possible to unfold some of the mechanisms of tumorigenesis which are evolutionary.

Materials and Methods

DNA sequence data and sequence alignment

The sequences for alignment were retrieved from the NCBI GenBank database (Benson *et al*., 2011) (available at www.ncbi.nlm.nih.gov) and saved in fasta file format for further analysis. These sequences were imported into the alignment explorer of MEGA version 4 software (Tamura *et al*., 2007). The ClustalW (Thompson *et al.*, 1994) algorithm was used to perform an initial multiple sequence alignment. This alignment was again manually edited and realigned using ClustalW with default parameters for Gap Opening, Gap Extension Penalty and DNA weight matrix to obtain optimal global sequence alignment. Phylogenetic trees were then built using this multiple sequence alignment file.

Phylogenetic tree building

Phylogeny tree building was carried out using MEGA version 4.0. The Neighbour-Joining (Saitou and Nei, 1987) method, which builds trees using a distance matrix, was chosen for phylogeny reconstruction of the sequences. Kimura 2-parameter (Kimura, 1980) distance model, which assumes uniform rate of substitution among sites, was selected as the nucleotide substitution model. To further increase the reliability of the phylogenetic tree obtained, 1000 Bootstrap replications were performed.

Functional divergence

During evolution many of the residues are subjected to functional constraints. Identification of these sites is important in studies that relate to understanding evolutionary history and can be estimated through functional divergence. In the current study, we calculated functional

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divergence between the various species for each gene using Diverge 1.04 software (Gu and Vander Velden, 1999). Protein sequences of all the species were retrieved from GenBank and were aligned using ClustalW in MEGA software using default parameters and this alignment was used as input for the Diverge software. Using this input, the software was used to build a p-distance Neighbour-Joining tree to delineate clusters. These clusters were then used to estimate statistical parameters such as site specific profile, which is useful to predict the amino acid residues which are vital for functional divergence. Residues estimated to have a functional divergence value greater than 0.2 were highlighted in the sequence alignment.

Table 1: Sequence details of p53.

S. No.	Organism	Common Name	Nucleotide Accession Number	Protein Accession Number
1.	*Homo sapiens*	Human	NM_000546	BAC16799
2.	*Cavia porcellus*	Domestic Guinea Pig	NM_001172740	NP_001166211
3.	*Macaca mulatta*	Rhesus Monkey	NM_001047151	AAB91534
4.	*Cercopithecus aethiops*	African Green Monkey	X16384	CAA34420
5.	*Macaca fascicularis*	Crab-Eating Macaque	AF456343	AAB91535
6.	*Sus scrofa*	Pig	NM_214145	NP_998989
7.	*Ovis aries*	Sheep	NM_001009403	ACP19318
8.	*Marmata monax*	Woodchuck	AJ001022	CAA04478
9.	*Delphinapterus leucas*	Beluga Whale	AF475081	AAL83290
10.	*Oryctolagus cuniculus*	Rabbit	NM_001082404	NP_001075873
11.	*Tupaia belangeri chinensis*	Chinese Tree Shrew	AF175893	AF175893
12.	*Bos indicus*	Zebu	U74486	AAB51214
13.	*Felis catus*	Domestic Cat	NM_001009294	BAA05653
14.	*Xenopus laevis*	African Clawed Frog	NM_001088098	CAA54672
15.	*Rattus norvegicus*	Norway Rat	NM_030989	NP_112251
16.	*Danio rerio*	Zebrafish	NM_131327	NP_571402
17.	*Bos Taurus*	Cattle	NM_174201	CAA57348
18.	*Oncorhynchus mykiss*	Rainbow Trout	NM_001124692	NP_001118164
19.	*Oryzias latipes*	Japanese Medaka	NM_001104742	AAC60146
20.	*Nothobranchius kuhntae*	Beira Killifish	EU391597	ACB30549
21.	*Meriones unguiculatus*	Mongolian Gerbil	AB033632	BAB69969
22.	*Mus musculus*	House Mouse	EU031806	AAA39883
23.	*Canis lupus familiaris*	Dog	AF060514	BAA78379
24.	*Cricetulus griseus*	Chinese Hamster	U50395	AAC53040
25.	*Xiphophorus maculates*	Southern Platyfish	AF043947	AAC31134
26.	*Tetraodon miurus*	Congo Puffer	AF071571	AAD34213
27.	*Platichthys flesus*	European Flounder	Y08919	CAA70123
28.	*Ictalurus punctatus*	Channel Catfish	AF074967	AAC26824
29.	*Barbus barbus*	Barbel	AF071570	AAD34212
30.	*Drosophila melanogaster*	Fruit Fly	DQ191318	NP_996268
31.	*Xenopus (Silurana) tropicalis*	Western Clawed Frog	NM_001001903	NP_001001903
32.	*Mesocricetus auratus*	Golden Hamster	U07182	AAB41344
33.	*Oreochromis niloticus*	Nile Tilapia	GU594898	ADE21938
34.	*Spalax judaei*	Blind Subterranean Mole Rat	AJ783406	CAH03844
35.	*Bombyx mori*	Silkworm	NM_001177410	NP_001170881
36.	*Entamoeba histolytica*		AJ489250	CAD32988
37.	*Mya arenaria*	Softshell	AF253323	ACK28179
38.	*Xiphophorus helleri*	Green Swordtail	AF043946	AAC31133
39.	*Coregonus lavaretus*	Common Whitefish	EU978857	ACH73252
40.	*Loligo forbesi*	Northern European Squid	U43595	AAA98563
41.	*Tigriopus japonicas*	Crustaceans	GQ327969	ADG86236
42.	*Homo sapiens (mutant p53)*		FJ207420	ACI25593
43.	*Rattus norvegicus (mutant p53)*		U90328	AAB80959

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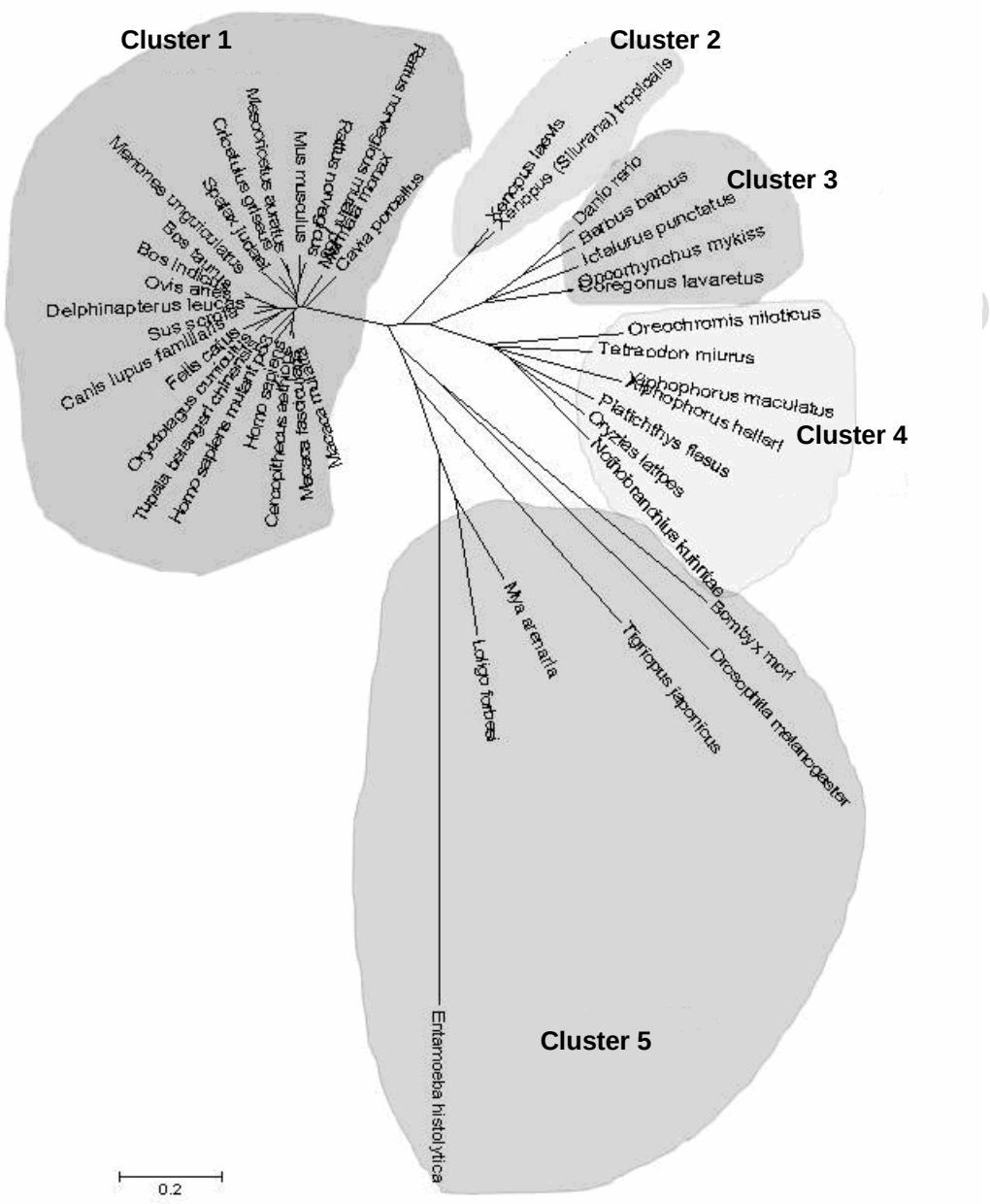

Figure 1: Phylogenetic tree of p53.

Results

We found that p53 and MDM2 showed some sequence similarity across the mammalian species, indicating that these genes probably regulate the normal cell division processes in these species as well. Details of the sequence alignment of p53 and MDM2 showing variable sites are presented in annexure-I and II.

Phylogenetic analysis of p53

A phylogenetic radiation tree constructed with p53 sequences of 41 species and a sequence each of mutant p53 in Humans and Rats, showed five clusters – a cluster consisting of mammals, a cluster of amphibians, two clusters with different species of fish, a fifth cluster of various other organisms. Information about the gene can be accessed in GeneCards database with GCid: GC17M007565

Phylogenetic analysis of MDM2

Phylogenetic reconstruction of thirteen sequences of MDM2 from various organisms showed five clusters - Humans along with six

other species formed the first cluster, Norway Rat and House Mouse form another cluster, Red Jungle Fowl forms an isolated cluster, a fourth cluster of Amphibians, another isolated cluster with Zebra fish. GeneCards database can be queried with GCid: GC12P069201 for information about the gene.

Table 2: Sequence details of MDM2.

S. No.	Organism	Common Name	Nucleotide Accession Number	Protein Accession Number
1.	*Canis lupus familiaris*	Dog	AB031276	BAB11975
2.	*Mus musculus*	House Mouse	U47934	AAB09031
3.	*Homo sapiens*	Human	GQ848196	ACX31156
4.	*Sus scrofa*	Pig	EU119401	ABV09038
5.	*Bos taurus*	Cattle	NM_001099107	NP_001092577
6.	*Equus caballus*	Horse	NM_001081844	NP_001075313
7.	*Felis catus*	Domestic Cat	NM_001009346	NP_001009346
8.	*Danio rerio*	Zebra fish	AF010255	AAB64176
9.	*Rattus norvegicus*	Norway Rat	NM_001108099	NP_001101569
10.	*Xenopus laevis*	African Clawed Frog	NM_001092601	NP_001086070
11.	*Gallus gallus*	Red Jungle Fowl	NM_001199384	NP_001186313
12.	*Xenopus (Silurana) tropicalis*	Western Clawed Frog	NM_203912	NP_989243
13.	*Pongo abelii*	Sumatran Orangutan	NM_001131213	NP_001124685

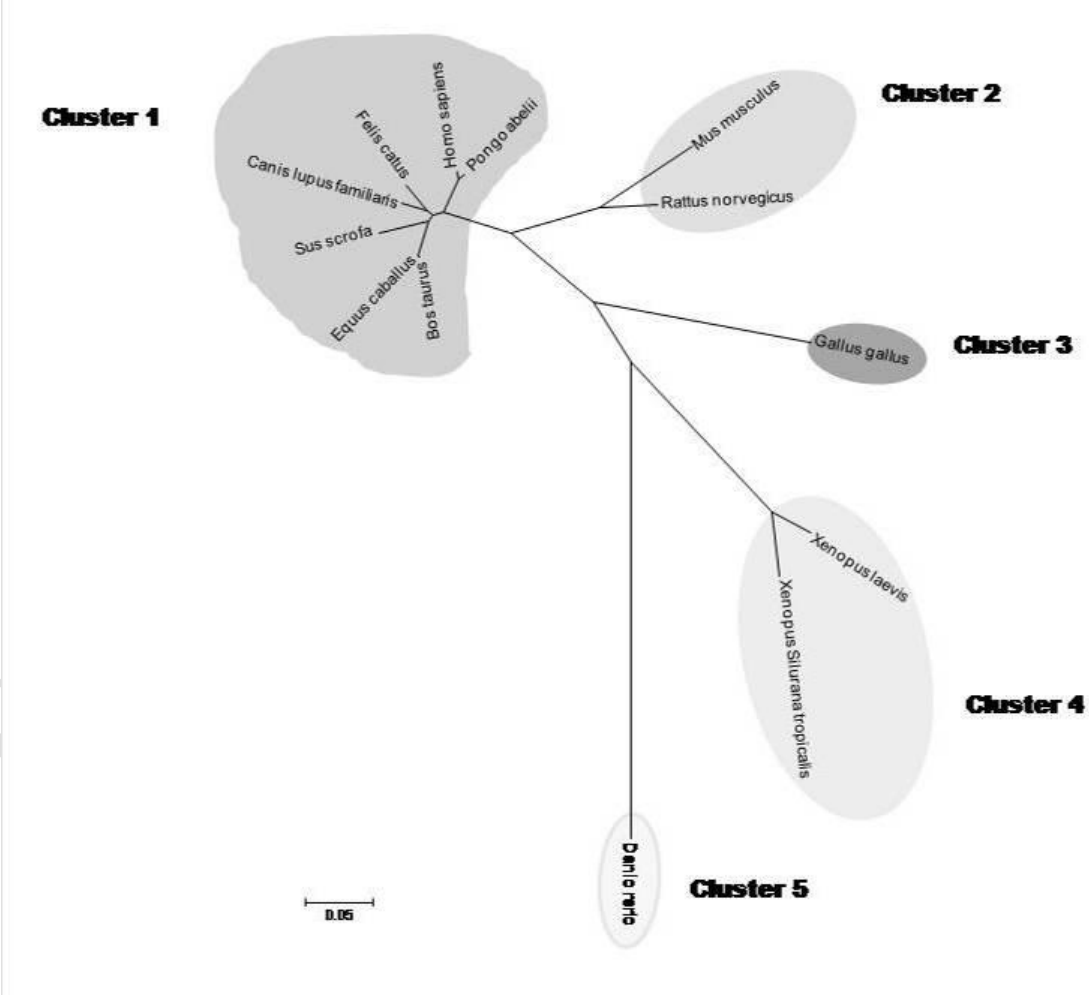

Figure 2: Phylogenetic tree of MDM2.

Functional divergence of p53 and MDM2

We used Diverge 1.04 to calculate the functional divergence of p53 and MDM2 proteins. Neighbour-Joining Tree with p-distance model was used for constructing trees to delineate clusters for both proteins. For the p53 protein, functional divergence was calculated by dividing the species into two clusters. The first cluster contains Mammalian species and the second cluster contains the rest of the organisms. The coefficient of functional divergence between cluster 1 and cluster 2 was 0.23. Further, using a posterior probability of 0.2 as a cutoff value we found 152 residues to be significantly divergent.

For the MDM2 protein also cluster 1 contained the Mammalian species and cluster 2 contained all other species. The coefficient of functional divergence between the two clusters was 0.1 and on applying a posterior probability value of 0.2, six functionally divergent residues were observed. In both the proteins, Mammalian sites were more or less similar to each other while differing from those in the second cluster.

Discussion

Neoplasia is characterized by accumulation of genetic abnormalities in the malignant cells. These changes lead to accumulation and proliferation of malignant cells and affect the patient's ability to fight infections. Though cure rates of 80% have been achieved, many patients still suffer due to therapy resistance and relapse (Pui *et al*., 2006; Pieters and Carroll, 2008; Bhojwani *et al*., 2006). p53 and MDM2 genes have been associated with treatment resistance and have been observed to have altered expression levels during relapse especially in leukemia which affect children. The p53 tumor suppression pathway is frequently deregulated in many cancers (Vogelstein *et al*., 2000). Since the genes in this pathway are functionally active in important cellular activities such as cell growth and proliferation, apoptosis, tumor suppression, they represent important targets to understand disease mechanisms. The key genes in this pathway, p53 and MDM2, have been observed to have a considerable effect in failure of chemotherapy in patients and hence the expression of these genes and their alterations need to be decoded. Phylogenetic studies are increasingly being used in recent years to understand the evolution of disease genes. The information obtained from phylogenetic study can further be used to construct better models of drug-gene interactions through homology modeling and affinity modeling (Sabitha *et al*., 2008). Studies conducted by Khan and Jamil (2010) on E2 ubiquitin enzymes, using phylogenetic approaches and through identification of sites under functional constraints, have reported that these enzymes are in a state of active evolution and have indicated that this information could be useful in understanding their role in neoplasia. Further, Shaik *et al.* (2009 a, b) carried out the phylogenetic analysis of genes related to the toxicological parameters and showed how this method could be useful in determining the evolutionary history of genes or even meta-analysis of polymorphic genes could be useful in predicting solid tumors.

In our study, we have used phylogenetic reconstruction methods to look at the evolutionary history of the p53 and MDM2 genes. A further study of evolutionary history can be done by analyzing the functional diversity of a protein/gene family. In our analysis for proteins of p53 and MDM2 genes, we obtained a coefficient of functional divergence (θ_{ML}) value greater than 0 indicating type I functional divergence. This implies that these select residues/sites are subjected to different evolutionary rates. Most of the sites in these proteins have a posterior probability less than 0.5, implying that there exists functional similarity across the two clusters. This functional similarity could help in identifying the exact mechanism of change which the genes encoding these proteins undergo, resulting in their association in the tumorigenesis process. We observed that these are largely conserved in the mammalian species; the presence of these genes in various species could indicate that these genes could have similar functions or could have evolved due to natural selection process. It is also possible that the presence of these genes in other lower species could indicate that similar disease conditions might exist in those species. A study of these genes in mammalian species that are similar to humans might help elucidate how these genes acquired the function of regulating the cell division.

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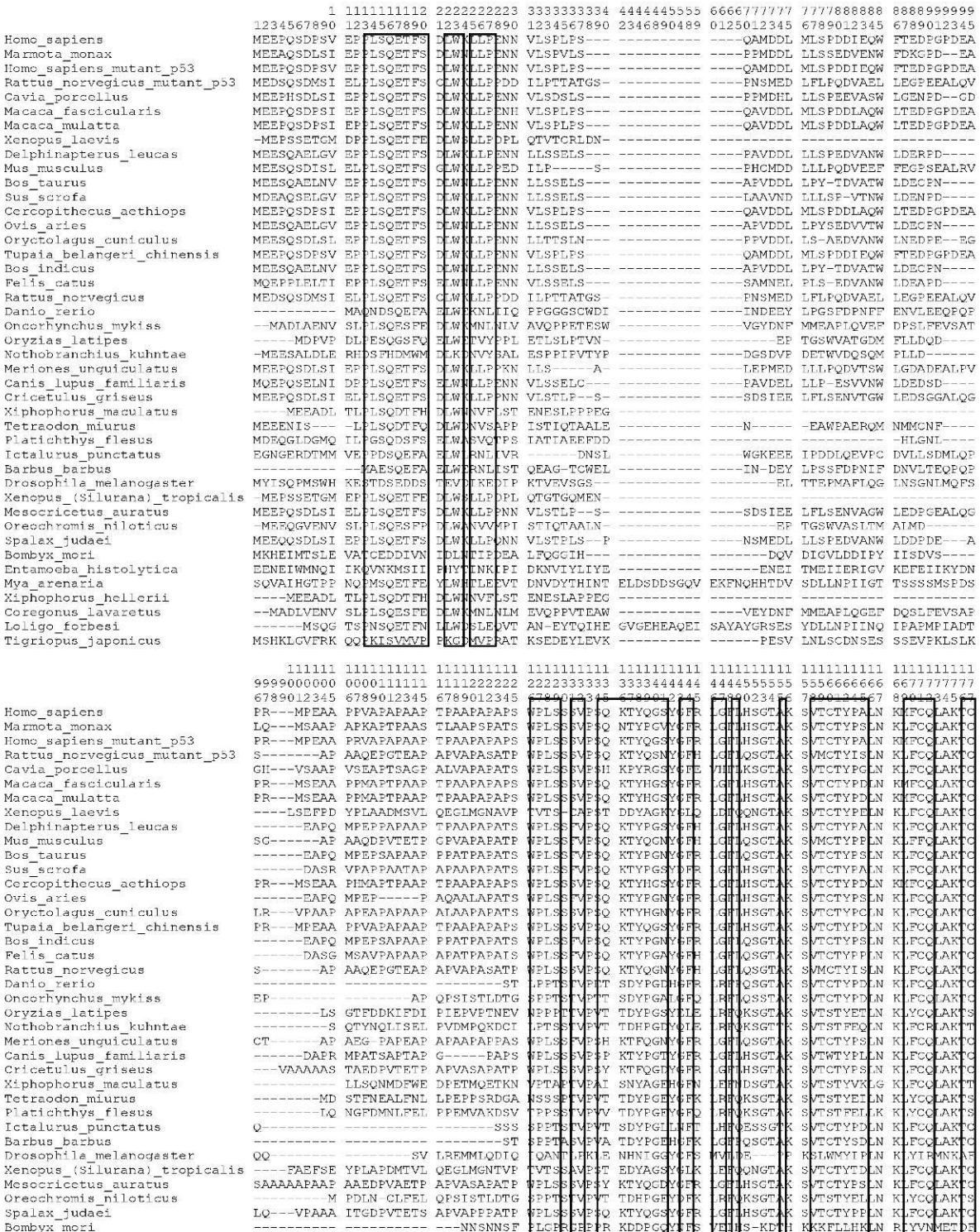
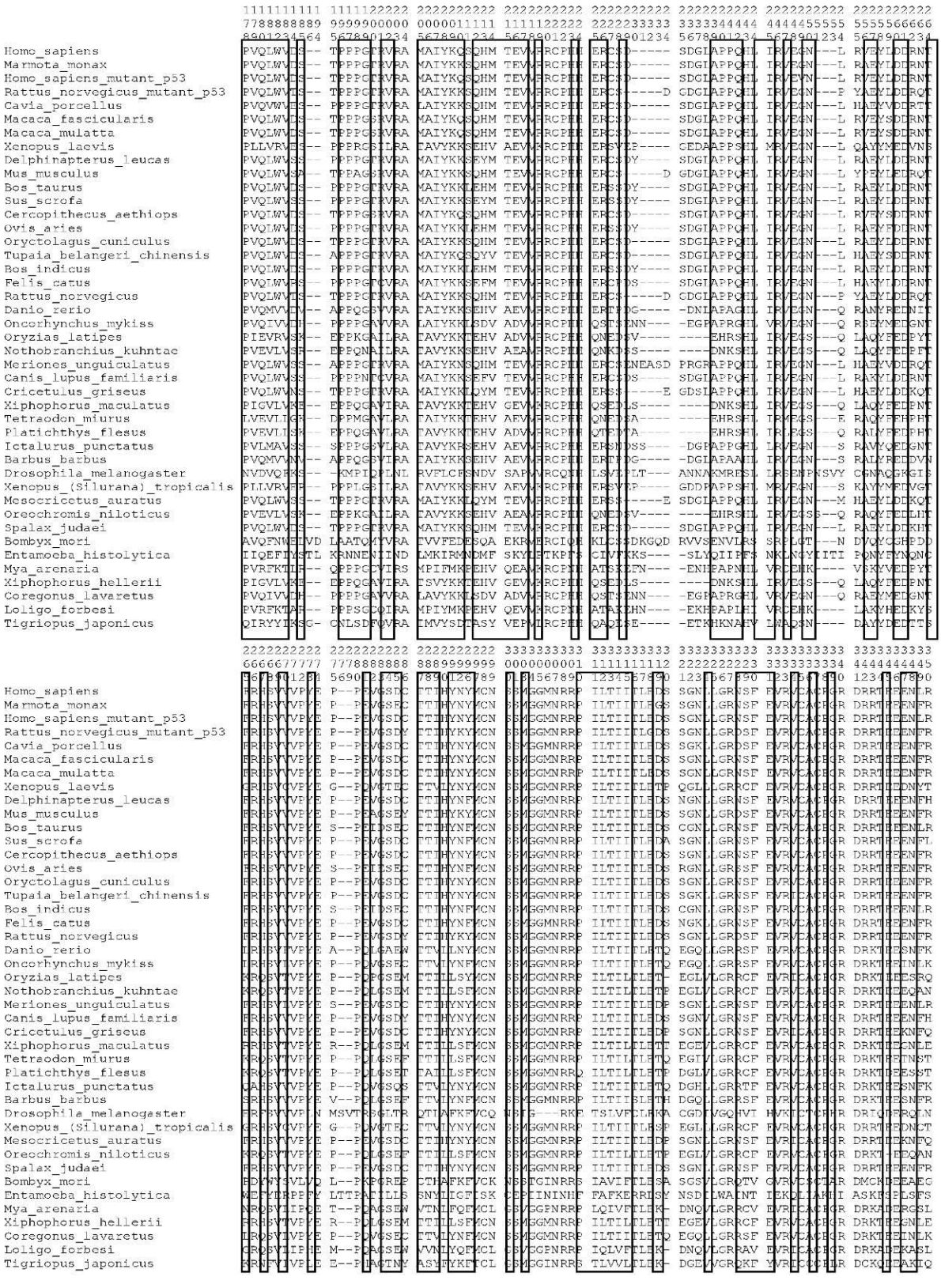
p53:




Homo_sapiens
Marmota_monax
Homo_sapiens_mutant_p53
Rattus_norvegicus_mutant_p53
Cavia_porcellus
Macaca_fascicularis
Macaca_mulatta
Xenopus_laevis
Delphinapterus_leucas
Mus_musculus
Bos_taurus
Sus_scrofa
Cercopithecus_aethiops
Ovis_aries
Oryctolagus_cuniculus
Tupaia_belangeri_chinensis
Bos_indicus
Felis_catus
Rattus_norvegicus
Danio_rerio
Oncorhynchus_mykiss
Oryzias_latipes
Nothobranchius_kuhntae
Meriones_unguiculatus
Canis_lupus_familiaris
Cricetulus_griseus
Xiphophorus_maculatus
Tetraodon_miurus
Platichthys_flesus
Ictalurus_punctatus
Barbus_barbus
Drosophila_melanogaster
Xenopus_(Silurana)_tropicalis
Mesocricetus_auratus
Oreochromis_niloticus
Spalax_judaei
Bombyx_mori
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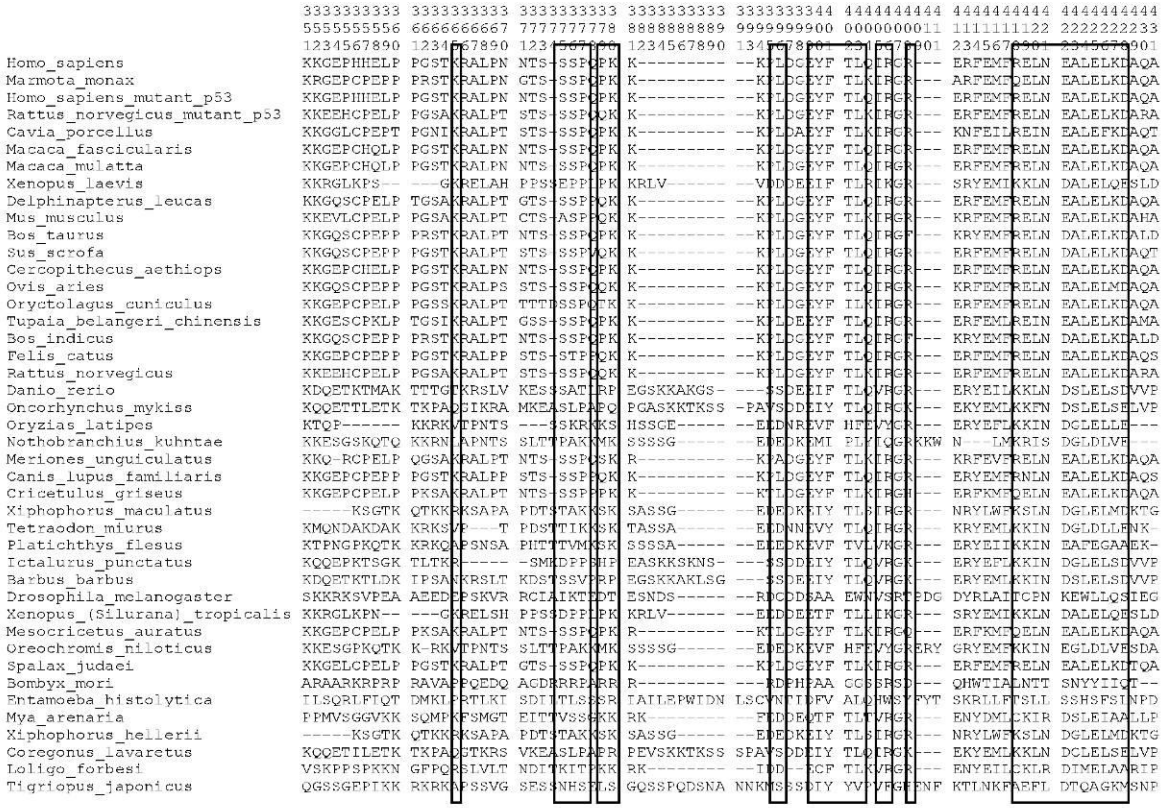
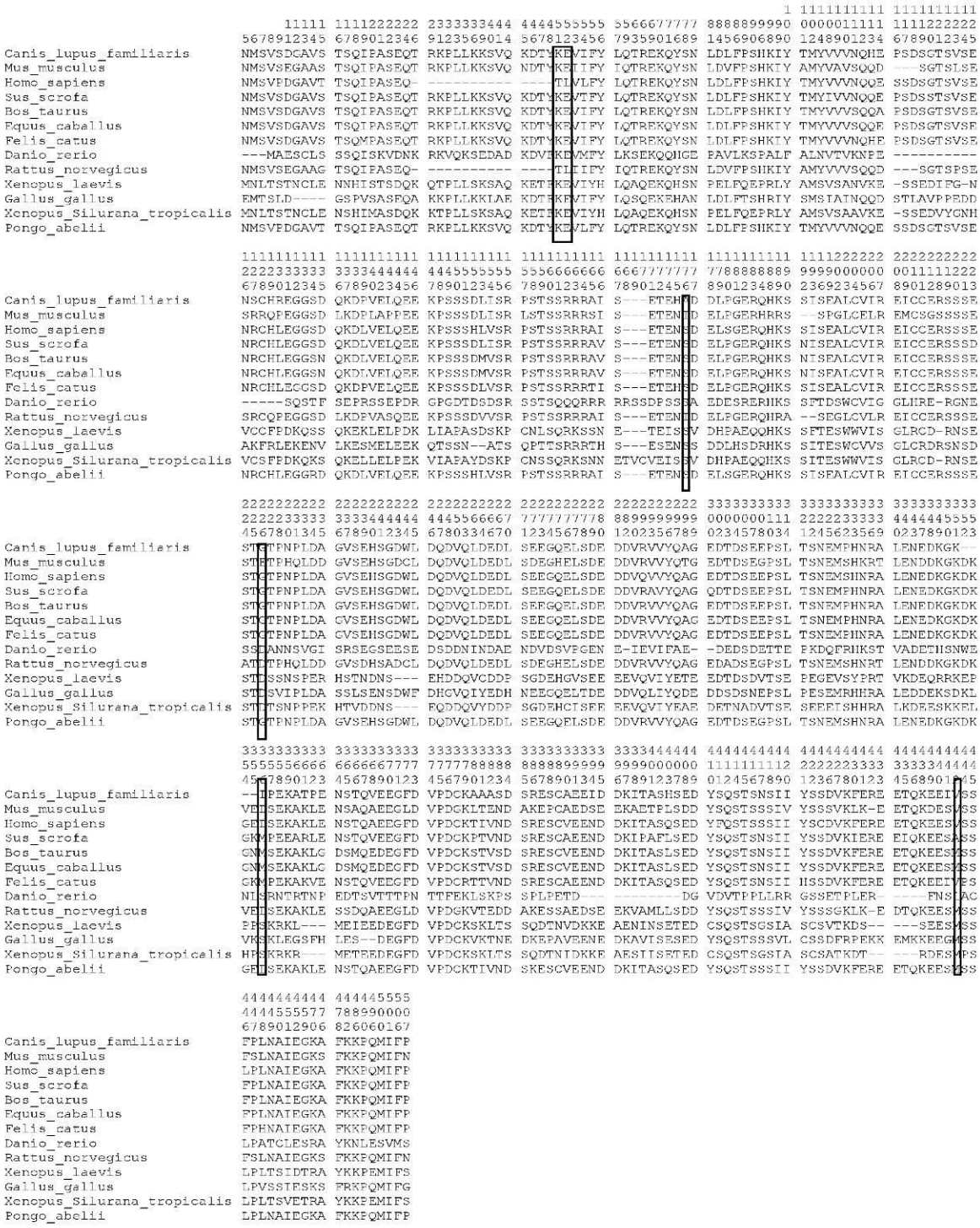
Loligo_forbesi
Tigriopus_japonicus



MDM2:



Apoptosis is a key process that is initiated to remove damaged cells from the system, thus ensuring the well-being of the species. Inhibition of this pathway due to alterations of genes that activate and regulate this pathway is a major characteristic feature of many malignancies. p53 is an important gene because of its close involvement in the activation of apoptotic pathways upon detection of damage. p53 activates the expression of its key regulator, MDM2 which in turn binds to p53 and regulates activity of p53 and so these two proteins control each other. Altered expression of either of these genes can thus have an inherent effect on cell death leading to uncontrolled cell proliferation and result in non-removal of damaged cells. The alterations of these genes also contribute to drug resistance since chemotherapeutic moieties function by activating apoptotic pathways to destroy leukemic cells.

Through phylogenetic study, we have determined that these genes share a high degree of sequence similarity across the Mammalian species and to a lesser extent with other animals, implying that they might share a common ancestor. Also, since they share sequence similarity it could be possible that the tumorigenic potential of these genes could be due to a buildup of changes during their evolution. This could entail that the mechanisms that lead to overexpression of these genes in solid tumors and leukemia could probably be deciphered by observing their genetic structure and function in the other species and by applying this information to human neoplasms. These data indicate the need to better understand not only how each of these gene are altered in disease but also how their interaction can contribute to malignancy. Interactions studies could also help determine why these genes play a more significant role in relapse than during initial disease development. This type of study could probably explore the design and development of drug moieties that could target the interaction of these two genes, instead of a single gene, and could thus prove to be an effective therapeutic strategy.

Conclusion

In conclusion, this is the first report of the phylogenetic analysis of p53 and MDM2 genes which revealed the sequence similarity shared with many species. The study of these two genes – oncogene (p53) and proto-oncogene (MDM2) - suggests that the mechanism of neoplasia is deeply rooted throughout evolution. This evolutionary history could help determine how these genes are altered in cancers and that MDM2-p53 interaction might play a very important role in the Tumorigenesis and Leukemogenesis processes during relapse in cancer patients. Thus, these molecules are indeed important for furthering our understanding of the cancer process.

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