RESEARCH ARTICLE

Overlapping Region of p53/Wrap53 Transcripts: Mutational Analysis and Sequence Similarity with microRNA-4732-5p

Nasser Pouladi[1,2], Shideh Montasser Kouhsari[1]*, Mohammadali Hosseinpour Feizi[3], Reyhaneh Ravanbakhsh Gavgani[3], Parvin Azarfam[3]

Abstract

Background: Although the majority of investigations concerned with TP53 and its protein have focused on coding regions, recently a set of studies highlighted significant roles of regulatory elements located in p53 mRNA, especially 5´ UTR. The wrap53α transcript is one of those that acts as a natural antisense agent, forming RNA-RNA hybrids with p53 mRNA and protecting it from degradation. Materials and Methods: In this study, we focused on the mutation status of exon 1α of the WRAP53 gene (according to exon 1 of p53) in 160 breast tumor tissue samples and conducted a bioinformatics search for probable miRNA binding site in the p53/wrap53 overlapping region. Mutations were detected, using single stranded conformation polymorphism (SSCP) and sequencing. We applied the miRBase database for prediction of miRNAs which target overlapping region of p53/wrap53 transcripts. Results: Our results showed all samples to have wild type alleles in exon 1 of TP53 gene. We could detect a novel and unreported intronic mutation (IVS1+56, G>C) outside overlapping regions of p53/wrap53 genes in breast cancer tissues and also predict the presence of a binding site for miR-4732-5p in the 5´ UTR of Wrap53 mRNA. Conclusions: From our findings we propose designing further studies focused on overexpression of miRNA-4732-5p and introducing different mutations in the overlapping region of wrap53 and p53 genes in order to study their effects on p53 and its ΔN isoform (Δ40p53) expression. The results may provide new pieces in the p53 targeting puzzle for cancer therapy.

Keywords: p53 - wrap53 - miRNA-4732-5p - mutation - 5´ UTR - cancer

Asian Pacific J Cancer Prev, **14 (6)**, 3503-3507

Introduction

The TP53 gene is one of the most studied and commonly mutated tumor suppressor genes in human cancers (Olivier et al., 2009). It encodes the p53 protein that triggers cell cycle arrest, senescence, or apoptosis in response to genotoxic and non-genotoxic stresses (Vilborg et al., 2010). p53 protein exerts multiple anti proliferative functions through the transcriptional control of many different target genes and through the protein-protein interaction (Olivier et al., 2009). Under normal condition, p53 has a key role in the prevention of human cancers (Farnebo et al., 2009). Its mRNA is ubiquitously expressed, but p53 protein is maintained at low levels in cells, due to rapid protein turnover. However, DNA damage and other stress signals trigger a significant increase in p53 protein half life and activate p53 as a transcription factor (Mahmoudi et al., 2009). p53 activities are critical to maintain genetic integrity and it must be tightly regulated to make certain of to ensure that its antiproliferative activities are induced in a controlled and timely manner (Olivier et al., 2009).

The murine/human double minute 2 (MDM2/HDM2) is an E3 ubiquitin ligase that promotes p53 degradation and forms a negative feedback loop that controls p53 activity (Aylon and Oren, 2007; Chen et al., 2010). In addition to ubiquitylation by MDM2, p53 is subjected to many other post-translational modifications in response to stresses. These modifications modulate the activity of p53, protein relocalization and stabilization, and its binding partners. The most studies of p53 regulation have focused on the p53 protein (Mahmoudi et al., 2009). Whereas recent efforts have revealed that p53 mRNA is an important regulatory target for many factors such as proteins and microRNAs. A number of RNA-binding proteins, such as HuR, L26, RPL26 and nucleolin, regulate p53 activity by binding the 5´ or 3´ UTR of p53 mRNA (Farnebo et al., 2009; Vilborg et al., 2010). Two miRNAs, miR-125a and miR125b, target a site within the 3´UTR of p53, resulting in a decrease in p53 mRNA and protein

*[1]Department of Cellular and Molecular Biology, School of Biology, College of Sciences, University of Tehran, [2]Department of Biology, Faculty of Science, Azarbaijan Shahid Madani University, [3]Department of Biology, Faculty of Natural Science, University of Tabriz, Tabriz, Iran *For correspondence: montasser20sh@khayam.ut.ac.ir, srna52@gmail.com*

level (Le et al., 2009; Zhang et al., 2009; Vilborg et al., 2010).

The discovery of a natural antisense transcript (NAT) of p53, WD repeat containing antisense to p53 (Wrap53), introduced a novel regulator of p53 and shed a new light on the responsible mechanisms of p53 posttranscriptional controls and other significant roles of noncoding exon 1 of p53 gene. Wrap53 gene is alternatively spliced and it has three alternative start exons: exon 1α, 1β and 1γ. Exon 1α directly overlaps the first exon of p53 in an antisense fashion and encodes Wrap53α transcript which forms RNA-RNA hybrid with p53 mRNA and protects it from degradation (Farnebo et al., 2010).

Accordingly it is postulated that the identification of mutations in 5´ UTR of the p53 gene, especially, overlapping region of TP53 and Wrap53 genes would help further definition of the mechanism of p53 gene regulation and provide new clues to the development of cancer. Also, recently it has been reported that the miRNAs can compete with antisense transcript and prevent the function of NAT-mediated gene regulation (Faghihi et al., 2010). Therefore, in this study, we were interested to focus on mutation status of exon 1 (i.e. 5´UTR) of p53 in breast tumor tissues and conducted a Bioinformatics search for a probable miRNA binding site in the p53/Wrap53 overlapping region and also predicted the presence of a binding site for miR-4732-5p in the 5´ UTR of Wrap53 mRNA.

Materials and Methods

Samples

A total of 160 breast cancer patients, without regard to family history, were selected for study. All patients had pathologically confirmed invasive ductal carcinoma. The mean age of the patients was 46/71 years. Tissues were collected immediately after surgery and transport to the laboratory in liquid nitrogen and stored at -80˚C prior to extraction of the DNA.

DNA extraction, PCR and SSCP

Genomic DNA was extracted from frozen tumor tissues by proteinase K digestion and the salting-out method. Two pairs of primers (A and B) were designed for amplification of p53 exon 1 (i.e. 5´UTR) in two partially overlapping amplicons and used in PCR as follows:

P1AF: 5´CAGCCCCAGCGATTTTCCCGA-3´
P1AR: 5´CAAAAGTCTAGAGCCACCGTCCAGG3´
P1BF: 5´CCGAACGCAAAGTGTCCCCGGA3´
P1BR: 5´TCT GCACCCTCCTCCCCAACTCCAT3´

Genomic DNA (0.1-0.5 μg) was amplified separately using 0.4 μM from Each set of Primers and PCR reactions with a total volume of 25 μl, containing 1X PCR buffer, 0.2mM dNTPs, 1.5 mM $MgCl_2$, 1U Taq polymerase. PCR was carried out by thermal cycler (Sensoquest, GmbH, Germany) at 35 cycles consisting of steps: denaturation at 95˚C for 5 minutes, annealing at 59˚C for P1A primer and 60˚C for P1B primer for 30 seconds and extension at 72˚C for 30 seconds in each cycle. The results were visualized using a 2% agarose gel and ethidium bromide staining. The PCR product was 201 bp for A primers and 242 bp for B primers.

The PCR product was mixed with SSCP loading dye (95% formamide, 10mM NaOH, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) at a ratio of 1:3. Denaturation was performed for 5 minutes at 96˚C. After denaturation, samples were cooled quickly on ice and then run immediately on a non-denaturating 12% polyacrylamide gel for 18 h at 100W. After electrophoresis, the DNA bands were visualized by silver staining (Figure 2).

Bioinformatics analysis

The miRNA registry, or miRBase, is a widely used database of miRNA sequences (Griffiths-Jones, 2006a; 2006b). We used this database for the prediction of miRNAs which target overlapping region of p53/Wrap53 transcripts. Sequences of mRNAs, particularly of their 5'-UTR, are often obtained from the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/). The minimum free energy (mfe) is calculated by the RNAhibrid tool (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/). Subsequently using ClustalW2, a multiple sequence alignment program, the sequences were aligned (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Results

In the present study, 320 chromosomes (160 cases of human breast cancer) have been analyzed for mutations in exon 1 of p53 tumor suppressor gene by PCR-SSCP. The complete sequence of exon 1 and the parts of its 5 and 3 flanking intronic regions were amplified in two partially overlapped amplicons, A (242 bp) and B (201 bp). Of all the patients that were analyzed, only one of the patients showed abnormal SSCP patterns in amplicon B (Figure 1a). The sequencing of this case revealed a novel and unreported mutation in intron 1 of p53 gene (IVS1+56, G>C) that is shown in Figure 1b and c. Our results showed that all of 320 chromosomes have wild type alleles in exon 1 of TP53 gene.

Our analysis of the miRNA database, mirbase, revealed

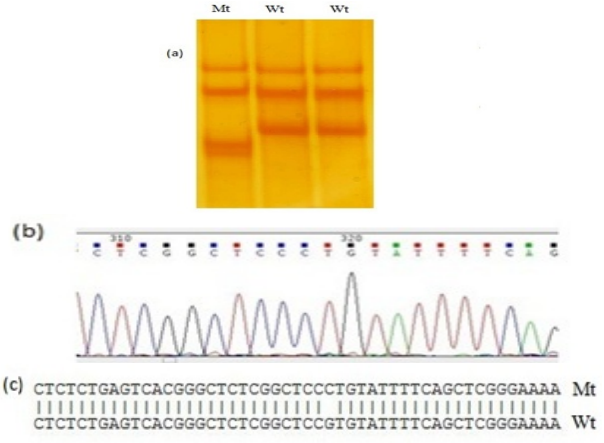

Figure 1. SSCP and Sequencing Results. SSCP analysis: (a) the patterns of the a Mt (mutant type) and two wt (wild type). (b) sequencing data showing homozigosity of CCG>CCC mutant in intron 1 of TP53 gene. (c) the sequence of mutant vs wild type

that hsa-miR-4732-5p has high sequence similarity with the part of 5´ UTR of p53 mRNA that overlaps Wrap53 transcript. Therefore, it has a binding site in Wrap53 mRNA. This binding site has a strong affinity to the miR-4732-5p (minimum free energy=-38 using RNAhybrid tools). The sequences of overlapping regions of p53 and Wrap53 transcripts (according to the review of Vilborg et al., 2010) and the sequence information of miR-4732 and its target site in Wrap53 mRNA have been shown in fig 2a and 2b respectively. Stem-loop hsa-miR-4732-5p has 76 nucleotides and is cleaved to generate mature miRNA with 23 nucleotides. 20 nucleotides of 23 can form RNA duplex with Wrap53 mRNA. Interestingly, we extracted p53/Wrap53 overlapping sequence data from NCBI reference sequences for comparative analysis between some species. These sequences were directly obtained from Wrap53 mRNA reference sequence for human, pan and macaca. The minus strand of the p53 mRNA sequence considered for Bose Taurus, mus musculus and Rattus norvegicus. As previously mentioned, ClustalW2 was used for aligning (Figure 3).

The target site of miR-4732 is shown in bold in human and compared with other mammals (Figure 3). 20 nucleotides which participate in mRNA/miRNA duplex have been completely conserved in human, pan and macaca but the similarity decreased toward rat until 50%. In other words, there is a striking variation in the degree of sequence conservation between human and rat. The GC content of overlapping region and the affinity of binding site in human and other species with miR-4732-5p (mfe) were shown in Table 1.

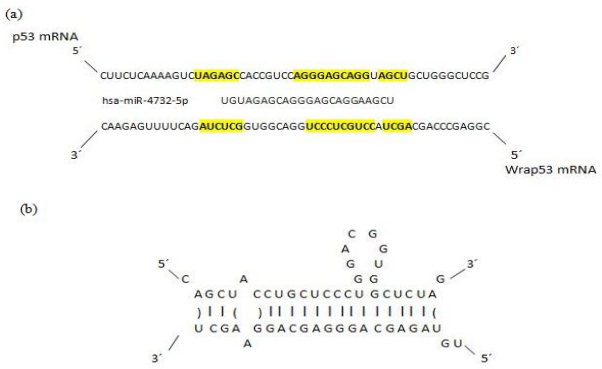

Figure 2. P53 and Wrap53 mRNAs, miR-4732-5p Sequences and their Binding Sites. (a) The sequences of overlapping regions of p53 and Wrap53 transcripts. (b) The sequence information of miR-4732-5p and its target site in Wrap53 mRNA

Figure 3. Alignment of Wrap53/p53 Overlapping Sequences. (H) Homo sapiens, (P) Pan troglodytes, (M) Macaca mulatta (B) Bos taurus (M) Mus musculus (R) Rattus norvegicus

Table 1. Comparison of Sequence Similarity, GC Content and the Affinity of Binding Site in Human and Other Species with miR-4732-5p

Mammals	Overlapping region GC content (%)	miRNA-binding site similarity with human (%)	mfe kcal/mol
Homo sapiens	60.4	100	-38.5
Pan troglodytes	60.4	100	-38.5
Macaca mulatta	60.4	100	-33.8
Bos taurus	64.0	80	-30.1
Mus musculus	50.0	65	-19.9
Rattus norvegicus	48.9	50	-19.3

Discussion

More than three decades have passed since the discovery of p53 and two decades since its identification as a tumor suppressor protein (Levine and Oren, 2009). Up to now, more than fifty thousand Pubmed results have been produced to shape our knowledge about p53 and its associated biology (Levine and Oren, 2009; Editorial, 2010). Despite the intense effort, many puzzles remain unresolved and novel findings add to the complexity of p53 network. Since p53 has crucial functions in controlling cell survival and death, it requires multiple layers of regulatory control to ensure correct temporal and spatial functions (Levine and Oren, 2009; Vilborg et al., 2010). This is achieved by variety of positive and negative regulators, often creating feedback loops. Three major levels of regulation are recognized: protein stability, protein activity, and subcellular distribution (Chatterjee and Pal., 2009). Although these levels have been extensively studied, increasing evidence shows that p53 mRNA is subjected to regulation that has an important impact on the p53 stress response.

The 5´ UTRs of vertebrate mRNAs typically contain various cis- regulatory elements such as the length of the 5´ UTR of mRNA, its thermal stability and GC content, the locations of secondary structures, multiple uAUGs (upstream AUG) and multiple uORFs (upstream ORF) which affect the translation efficiency of the main coding sequence (CDS) of the transcript (Takagi et al., 2005; Crowe et al., 2006). These elements may be considered as binding sites of regulatory proteins. Furthermore, mutations occurring in these regulatory sequences, influence gene expression at the level of translation (reviewed by Chatterjee and Pal, 2009).

Although the majority of investigations concerned with TP53 and its protein have focused on coding region, recently a set of studies highlighted the significant role of cis-reulatory elements located in p53 mRNA, especially 5´ UTR. The p53 5´ UTR can regulate both translation and stability of the p53 mRNA. For example, Takagi et al identified two proteins regulating the translation of p53 mRNA: RPL26 enhances p53 expression whereas nucleolin represses it in response to DNA damage by Ionizing Radiations. These proteins are implicated in the folding and the stabilization of 5'UTR secondary structures (Takagi et al., 2005). More recently, Discovery of a natural antisense transcript of p53, Wrap53, introduced a novel regulator of p53 and shed a new light

on the responsible mechanisms for p53 posttranscriptional controls (Mahmoudi et al., 2009). They showed that the Wrap53 gene located on the chromosome 17, directly overlaps the first exon of p53 (encoding the p53 5´ UTR) in a sense-antisense fashion and encodes WRAP53α transcript which forms RNA-RNA hybrid with p53 mRNA and protects it from degradation. It may be the most surprising part of the 5´ end of p53 mRNA story that has been started by Bienz et al in 1984-1985 (Bienz et al., 1984; Bienz-Tadmor, 1985; Chatterjee and Pal, 2009). Undoubtedly it would not be the end of this story and the appearance of new concepts and players in mechanisms of gene regulation, such as non-coding RNAs, will fulfill the role of cis-regulatory elements located in 5´ UTR of p53 mRNA.

The discovery of Wrap53 revealed the role of NAT-mediated gene regulation of p53 protein expression. It is clear that disrupting of sense-antisense hybrid by occurring of mutations or targeting through endogenous small RNAs can affect this regulation mechanism. Multiple lines of evidence suggest that mutations and single nucleotide polymorphism (SNP) in UTRs influence expression of specific genes at the level of translation (Chatterjee and Pal, 2009). Mutations/or polymorphisms in the 5´UTR of BRCA1 and BRCA2 genes and the androgen receptor gene have been reported to be involved in susceptibility of breast and prostate cancers, respectively (Crocitto et al., 1997; Signori et al., 2001; Gochhait et al., 2007). Also, Chen and Kastan (2010) showed that the creation of mutation as few as 3 bases in either of the two complementary UTR sequences abolishes the binding of RPL26 to human p53 mRNA and stimulates p53 translation.

More recently, Grover et al. (2011) suggested that mutations occurring in the p53 IRES might have profound implications for de-regulation of its expression and activity.

As previously mentioned, we believed that the identification of mutations in 5´ UTR of the p53 gene, especially, overlapping region of TP53 and wrap53 genes would help further define the mechanism of p53 gene regulation. Analysis of the IARC p53 database (version R15; wwwp53.iarc.fr) represents the absence of submitted mutations in non-coding exon 1 of p53. Nonetheless, almost all of the screening and sequencing studies, used in this database, are designed for detection of coding and some intronic regions. Also, Alonso et al. (2009) resequenced 5´ UTR of TP53 (224 bp long) in a sample of 116 chromosomes, including healthy Europeans, European melanoma patients, Africans, Asians and Australian Aborigines, no SNP was found within this region. In this study we could only detect a novel and unreported intronic mutation in outside of overlapping regions of p53/Wrap53 genes in breast cancer tissues. Despite these data, together with the studies described above, underscore high conservation and immutability of 5´ UTR of p53, further research is needed to confirm these results because, more recently, khan D et al. reported for the first time that SNP in the 5' UTR of the p53 MRNA might have a role in translational control of p53 tumor-suppressor gene (Khan et al., 2012).

Interestingly, our analysis of the miRNA database, mirbase, revealed that hsa-miR-4732-5p has high sequence similarity with the part of 5´ UTR of p53 mRNA that overlaps wrap53 transcript. Therefore, it has a binding site in wrap53 mRNA. This binding site has a strong affinity to the miR-4732-5p (minimum free energy=-38 using RNAhybrid tools). Our results suggest that covering the 5´ UTR of the wrap53α transcript by hsa-miR-4732-5p miRNA might block Wrap53/p53 hybrid formation and reduce p53 abundance. Recently, Fagihi et al. (2010) showed that BACE1- antisense transcript and miR-485-5p ncRNAs compete for binding to the sixth exonic region of BACE1 mRNA (Faghihi, 2010). Therefore, it could be extrapolated to the p53/Wrap53 sense-antisense pairs and miR-4732-5p. Also, Persson et al. (2011) reported that miR-4732-5p locates in regions which amplify in breast cancer (cytoband: 17q11.2). Since they could not detect miRNA-4732-5p in any other tissues except breast, this suggests that it may be specific to breast (Persson et al., 2011).

Since the tumor suppressor p53 has a central role in therapeutic modality, in recent years, considerable efforts were focused on new concepts and mechanisms related to regulation of p53 expression such as: the role of mutant p53 in human cancer and drug resistance, emerging of miRNAs as new players in gene regulation and wrap53 as a novel regulator of p53.

Recently several lines of evidences have shown that wrap53 is upregulated by some therapeutic agents such as idarubicin, etoposid and cisplatein (Bug and Dobbelstein, 2011; Yuan et al., 2011). Also it is going to be a new biomarker for cancer diagnosis (Zhang et al., 2012). Undoubtedly, identification of endogenous small interfering RNAs (endo-siRNA) or miRNAs that can target sense/antisense hybrid of wrap53/p53 transcripts would elucidate a role of NAT-mediated gene regulation in the p53 pathway. Our results suggest that miRNA-4732-5p can be a potent candidate for disrupting of this sense/antisense hybrid that must be confirmed.

Overall, recent studies address to accumulation of cis-regulatory and trans-regulatory elements on 5´ UTR of p53 mRNA and complexity of their interactions. Therefore, it is necessary to design multidisciplinary experiments tackling this challenge. Our results propose designing further studies focused on the overexpression of miRNA-4732-5p and introducing of different mutations into the overlapping region of wrap53 and p53 genes in order to study of their effects on p53 and its ΔN isoform (Δ40p53) expression. The results may be new pieces in p53 targeting puzzle in cancer therapy.

References

Alonso S, Izagirre N, López S, et al (2010). The diversity profile of TP53 is influenced by positive selection on the immediately upstream locus WDR79. *Hum Hered*, **69**, 34-44.

Aylon Y, Oren M (2007). New plays in the p53 theater. *Current Opinion in genetics Development*, **21**, 86-92.

Bienz B, Zakut-Houri R, Givol D, Oren M (1984). Analysis of the gene coding for the murine cellular tumor antigen p53. *EMBO J*, **3**, 2179-2183.

Bienz-Tadmor B, Zakut-Houri R, Libresco S, et al (1985). The 5' region of the p53 gene: evolutionary conservation and

evidence for a negative regulatory element. *EMBO J*, **4**, 3209-13.

Bug M, Dobbelstein M (2011). Anthracyclines induce the accumulation of mutant p53 through E2F1-dependent and -independent mechanisms. *Oncogene*, **30**, 3612-3624.

Chatterjee S, Pal JK (2009). Role of 5´ and 3´ untranslated regions of mRNAs in human diseases. *Biol Cell*, **101**, 251-62.

Chen F, Wang W, El-Deiry WS (2010). Current strategies to target p53 in cancer. *Biochem Pharmacol*, **80**, 724-30.

Chen J, Kastan MB (2010). 5'-3'-UTR interactions regulate p53 mRNA translation and provide a target for modulating p53 induction after DNA damage. *Genes Dev*, **24**, 2146-56.

Crocitto LE, Henderson BE, Coetzee GA (1997). Identification of two germline point mutations in the 5'UTR of the androgen receptor gene in men with prostate cancer. *J Urol*, **158**, 1599-601.

Crowe ML, Wang XQ, Rothnagel JA (2006). Evidence for conservation and selection of upstream open reading frames suggests probable encoding of bioactive peptides. *BMC Genomics*, **26**, 7-16.

Editorial (2010). Drug discovery in the p53 field. *Seminars Cancer Biology*, **20**, 1-2.

Faghihi MA, Zhang M, Huang J, et al (2010). Evidence for natural antisense transcript-mediated inhibition of microRNA function. *Genome Bio,* **11**, 56.

Farnebo M (2009). Wrap53, a novel regulatory of p53. *Cell Cycle*, **8**, 2343-6.

Farnebo M, Bykov V JN, Wiman K (2010). The p53 tumor suppressor: A master regulator of diverse cellular processes and therapeutic target in cancer. *Biochem Biophys Res Communications*, **396**, 85-9.

Gochhait S, Bukhari SIA, Bairwa N, et al (2007). Implication of BRCA2 -26G>A 5' untranslated region polymorphism in susceptibility to sporadic breast cancer and its modulation by p53 codon 72 Arg>Pro polymorphism. *Breast Cancer Res*, **9**, 71.

Griffiths-Jones S, Grocock RJ, Van Dongen S, Bateman A, Enright AJ (2006a). The microRNA sequence database. *Methods Molecular Biology*, **342**, 129-38.

Griffiths-Jones S, Grocock RJ, van Dongen S, et al (2006b). miRBase: microRNA sequences, targets, and gene nomenclature. *Nucleic Acids Res*, **34**,140-4.

Grover R, Sharathchandra A, Ponnuswamy A, et al (2011). Effect of mutations on the p53 IRES RNA structure Implications for de-regulation of the synthesis of p53 isoforms. *RNA Biology*, **8**, 132-42.

Khan D, Sharathchandra A, Ponnuswamy A, et al (2012). Effect of a natural mutation in the 5' untranslated region on the translational control of p53 mRNA. *Oncogene*, [Epub ahead of print].

Le MT, Teh C, Shyh-Chang N, et al (2009). MicroRNA 125b is a novel negative regulator of p53. *Genes Dev*, **23**, 862-76.

Levine AJ, Oren M (2009). The first 30 years of p53: growing ever more complex. *Nature Reviews Cancer*, **9**, 749-58

Mahmoudi S, Henriksson S, Corcoran M, et al (2009). Wrap53, a natural p53 antisense transcript required for p53 induction upon DNA damage. *Molecular Cell*, **33**, 462-71.

Olivier M, Petitjean A, Marcel V, et al (2009). Recent advances in p53 research: an interdisciplinary perspective. *Cancer Gene Therapy*, **16**, 1-12.

Persson H, Kvist A, Rego N, et al (2011). Identification of new microRNAs in paired normal and tumor breast tissue suggests a dual role for the ERBB2/Her2 gene. *Cancer Res*, **71**, 78-86.

Signori E, Bagni C, Papa S, et al (2001). A somatic mutation in the 5'UTR of the BRCA1 gene in sporadic breast cancer causes down-modulation of translation efficiency. *Oncogene*, **20**, 4596-600.

Takagi M, Absalon MJ, McLure KG, Kastan MB (2005). Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell*, **123**, 49-63.

Vilborg A, Wilhelm A, Wiman K (2010). Regulation of tumor suppressor p53 at the RNA level. *J MOL MED*, **88**, 645-52.

Yuan JM, Li XD, Liu ZY, et al (2011). Cisplatin induces apoptosis via upregulating Wrap53 in U-2OS osteosarcoma cells. *Asian Pac J Cancer Prev*, **12**, 3465-9.

Zhang H, Wang D, Mahmoudi S, et al (2012). WRAP53 is an independent prognostic factor in rectal cancer - a study of Swedish clinical trial of preoperative radiotherapy in rectal cancer patients. *BMC Cancer*, **12**, 294.

Zhang Y, Gao JS, Tang X, et al (2009). MicroRNA 125a and its regulation of the p53 tumor suppressor gene. *FEBS Lett*, **583**, 3725-30