A Single Nucleotide Polymorphism in the *MDM2* Gene: From a Molecular and Cellular Explanation to Clinical Effect

Gareth L. Bond, Wenwei Hu, and Arnold Levine

The Cancer Institute of New Jersey, Robert Wood Johnson Medical School, New Brunswick, New Jersey and The Institute for Advanced Studies, Princeton, New Jersey

Abstract

In a recent article, a candidate pathway approach was taken to try to identify single nucleotide polymorphisms (SNP) that make up the genetic variation, which underlies the phenotypic variation seen between individuals in their susceptibility to cancer and the progression of their disease. The p53 stress response pathway was chosen given its well-documented importance in tumor suppression. A SNP was found which associates with the attenuation of the p53 pathway and the acceleration of tumor formation in humans and data was presented which describe a molecular mechanism for these phenotypes. (Cancer Res 2005; 65(13): 5481-4)

In 2001, the sequence of the human genome was completed and it became clear that different individuals were >99% identical (1, 2). Among the differences between people were about 4.5 million single nucleotide polymorphisms (SNP) distributed throughout the genome, in coding and noncoding regions, and these differences could contribute to many of the individual traits that define us as unique. This includes predisposition to diseases, responses to drugs or therapies, as well as interactions with known mutations that predispose patients to diseases. One of the difficulties that these observations create is how to choose which SNP, out of such a large number of candidates, should be tested for correlations with a specific disease? In the field of cancer research, the past 30 years have led to the identification of oncogenes and tumor suppressor genes and the elucidation of the signal transduction pathways in which they reside. Different cancers often harbor germ line and somatic mutations in selected genes in those pathways that alter the homeostatic mechanisms of cells so as to permit inappropriate cell division and block programmed cell death. These mutations have identified which signal transduction pathways play a role in the origins of human cancers and studies elucidating the functions of these networks have given us the molecular and cellular explanations for the origins of cancers. This has led to the obvious conclusion that SNPs in these pathways that are often altered in cancerous cells could well be good candidates for having an effect upon either the frequency of cancer in a population, the age of onset of a cancer in an individual, or the responses to treatment of cancers. This might be termed the SNP candidate pathway approach for identifying SNPs that affect cancers.

We have chosen to carry out this exercise with the p53 pathway whose function is to respond to a wide variety of stress signals. Among these stresses are DNA damage, telomere shortening, hypoxia, low levels of ribosome biogenesis, low levels of ribonucleoside triphosphates, inflammation and nitric oxide signaling, cold and heat shock, mitotic spindle damage, and even the mutational activation of selected oncogenes (Rb-E2F-1, myc, ras, and β-catenin; ref. 3). The occurrence of one or more of these stress signals is associated with the chemical modification of the p53 protein (phosphorylation, acetylation, methylation, ubiquitination, neddylation, or summolation) and a dramatic increase in the half-life of the p53 protein (4). The p53 protein concentration increases in a cell and it becomes an active transcription factor. This is mediated, at least in part, by inactivating a key negative regulator of p53 (i.e., Mdm2). Mdm2 is the major p53 protein ubiquitin ligase responsible for inhibiting p53 activity and promoting its degradation (5). The p53 transcription factor then binds to a specific set of DNA sequences that regulate the transcription rates of p53-responsive genes that at least in part implement the responses to these stress signals (6). There are three major outcomes of the p53 stress response: (a) cell cycle arrest, (b) cellular senescence, and (c) apoptosis. The purpose of this signal transduction pathway is to ensure the fidelity of the duplication process of DNA in the cell. Stress, such as DNA damage, increases the error rate for the duplication of DNA; thus, cell cycle arrest provides the time to repair the DNA before duplication, whereas senescence and apoptosis eliminate clones of cells that would otherwise propagate with a high error rate.

This signal transduction pathway is composed of >100 genes, many of which have SNPs in their coding or regulatory regions, and we might expect that some of these SNPs will make the output of the pathway more or less efficient. For example, in the *p53* gene itself, there is a SNP at codon 72 that encodes either proline or arginine and its frequency in the population varies from the equator to higher latitudes suggesting a selection pressure upon these two forms of p53 protein (7). Indeed, several lines of evidence suggest that this polymorphism can play a role in apoptosis and cancer formation in humans (8, 9). In 82 genes in the p53 pathway, we have identified 1,335 SNPs in the noncoding (n = 977) and coding regions (n = 358). In the p53 signal transduction network, both the *p53* and *MDM2* genes form central nodes with many inputs and outputs that connect almost every function of the pathway (3, 10). The levels of the MDM2 protein in a cell or organism seem to have a large effect upon the p53 response and cancer formation (11). In mice that produce reduced levels of the MDM2 protein, the offspring are small, lymphopenic, and radiosensitive with increased rates of apoptosis in both lymphocytes and epithelial cells and these phenotypes are dependent upon p53 functions (12). Crossing these mice with transgenic mice prone to developing lymphomas reduces the incidence of lymphomas, showing the role of p53 and MDM2 in these cancers in animals (13). Similarly, the overexpression of MDM2 in mice results in the production of tumors with as little as a 4-fold increase in MDM2 levels giving rise to tumors in 100% of the mice (14). In humans, the *MDM2* gene is amplified in about 30% of the osteosarcomas and soft tissue sarcomas (15–17). In a study by Momand et al., they

Requests for reprints: Arnold J. Levine, Institute for Advanced Study, School of Natural Sciences, Einstein Drive, Princeton, NJ 08540. Phone: 212-327-8080; Fax: 1-212-327-8900; E-mail: alevine@ias.edu.

showed that 7% of 3,000 tumors screened from 28 different tumor types had MDM2 amplifications (18). When those tumors with either p53 or MDM2 mutations were studied, 65% had p53 mutations, 35% had MDM2 mutations, and only 4% had both mutations, suggesting that the amplification of the *MDM2* gene functions to inactivate p53. Thus, there is ample evidence in both mice and humans that small increases in the levels of the MDM2 protein can attenuate the p53 function leading to cancer formation.

The *MDM2* gene in mice and humans seems to have two promoter-enhancer regions that regulate the levels of MDM2 mRNA. The first promoter is 5′ to the first exon and likely regulates the basal level of MDM2 in a nonstressed cell. The second promoter region is in the first intron (in the *MDM2* gene, the third exon is the first coding exon) and this region contains both an AP1-Ets and a p53-responsive DNA sequence that increases the expression of MDM2 after a p53 response (19, 20). This intron is composed of 524 nucleotides in humans and SNPs have been detected in two positions in this sequence; at nucleotide 309, there is a T-to-G change and at 344 a T-to-A change (the convention is to indicate the change from the base at the highest frequency in the population to the lower frequency; ref. 21). Several computer algorithms that identify transcription factor binding sites indicated that SNP309, the T-to-G change, extended the length of a putative SP1 binding site. This was confirmed *in vitro* by carrying out gel shift experiments with purified SP1 protein and oligonucleotides containing either the major allele or SNP309. The SP1 protein bound about 4-fold better to the SNP309 oligonucleotide than to the major allele. Thus, it seemed that SNP309 could create an improved SP1 site. Small interfering RNA directed against SP1 reduced the levels of SP1 (and not SP3) in cells and this lowered the levels of a known SP1-regulated gene (*cyclin D1*) and MDM2 but only in cells that were homozygous for SNP309 (G/G) and not in cells that were T/T at the *SNP309* locus. Indeed a drug, mithramycin A, which binds tightly to SP1 DNA sites and inhibits the transcription of genes regulated by SP1, preferentially blocked the synthesis of MDM2 in G/G SNP309 cells when compared with MDM2 synthesis in T/T cells. Thus, SNP309 (G/G) creates an improved SP1 site that regulates the basal levels of MDM2 transcription only in these cells and not in T/T cells. A survey of cell lines with SNP309 (G/G) and with the major allele (T/T) shown at the RNA and the protein levels that SNP309 increased the basal levels of MDM2 in those cells with SNP309. The higher levels of MDM2 in SNP309 containing cells had a functional consequence. Those cells with SNP309 and a higher level of MDM2 proteins had a lower apoptotic response than cells that were T/T at the *SNP309* locus. The apoptotic response is measured by determining the percentage of cells in the cell line that undergo apoptosis after DNA damage (in a p53 wild-type cell). Similarly, in cells with the *G/G SNP309* alleles, the levels of mRNA from genes that are regulated by p53 after a DNA damage response were much lower than the levels of mRNA from these same genes in a cell line that was T/T at the *SNP309* locus. Most interesting, the SNP309 inhibition of p53 mediated apoptosis could be reversed 2- to 3-fold by treatment of these SNP309 cells with mithramycin A that inhibits the SP1 function in those cells and lowers the MDM2 levels. The higher levels of MDM2 in SNP309 cells attenuate the increases in p53 protein levels observed after cells suffer DNA damage so that T/T cells can increase p53 protein levels 5- to 14-fold after a stress signal, whereas SNP309 (G/G) cells showed 2- to 3-fold increases in p53 levels. Clearly, the higher basal levels of MDM2 in cells can have a functional consequence in reducing p53 levels, p53-mediated transcription of genes, and apoptosis of those cells after DNA damage; thus, a higher percentage of cells under stress will live and propagate if they have a SNP309 (G/G) genotype (21).

Table 1. Age of cancer onset and SNP309

	Average age of onset	Median age of onset	
T/T	27	27	All LF, tumor types
SNP309	20	18	
T/T	22	14	LF, soft tissue sarcomas
SNP309	10	2	
T/T	36	39	LF, breast cancer
SNP309	30	29	
T/T	57	59	Sporadic soft tissue sarcomas
G/G	45	38	

Abbreviation: LF, Li-Fraumeni.

The experiments described above were carried out with transformed or cancerous cell lines in culture. When nontransformed or normal cells are treated with DNA-damaging agents in cell culture they usually undergo a p53 response that results in cell cycle arrest. To examine the effect of SNP309 in nontransformed cell lines, fibroblasts were obtained from individuals with Li-Fraumeni syndrome who carry a p53 germ line mutation in one allele and thus have half the dosage of wild-type p53 activity. These cell lines were typed for SNP309 and two were T/T homozygotes and one was a G/G homozygote. These three cell lines were treated with a DNA-damaging agent and both of the T/T cell lines underwent a cell cycle arrest in G_2, as measured by the accumulation of cells with tetraploid DNA content. In contrast, the G/G cells did not show such a response. In fact, no significant difference in the DNA content of the SNP309 homozygous cells was seen. A failure to arrest division after DNA damage results in the propagation of many mutations and a cancer prone phenotype. Thus, nontransformed cells in culture also have an attenuated p53 response if they are SNP309 (G/G) homozygous when compared with cells that are T/T at that locus. It is useful to point out here that all three cell lines under study here have many genetic differences not only at the SNP309 locus; thus, these studies show correlations with very reasonable molecular and cellular explanations, which strengthen the conclusions.

Having characterized the SNP309 phenotype *in vitro* and cell culture, we next tested these ideas with populations of patients with cancers. In collaboration with Louise Strong, Linwah Yip, Shih-Jen Huang, and Guillermina Lozano at the M.D. Anderson Cancer Center, 88 individuals with Li- Fraumini syndrome who each carried a p53 germ line inactivating mutation in one allele were typed for SNP309. The G and T allele frequencies for SNP309 in this population (176 chromosomes typed) were similar to the allele frequencies we have observed in laboratory volunteers (110 chromosomes): 12% G/G, 40% G/T, 48% T/T. Of the 88 Li-Fraumeni patients, 66 had already been diagnosed with at least one cancer with the most common cancers being soft tissue sarcomas (n = 20), breast cancer (n = 17), and osteosarcomas (n = 13). When we examined the age of onset of any type of cancer in the 66 patients, the average age of onset for the T/T genotype

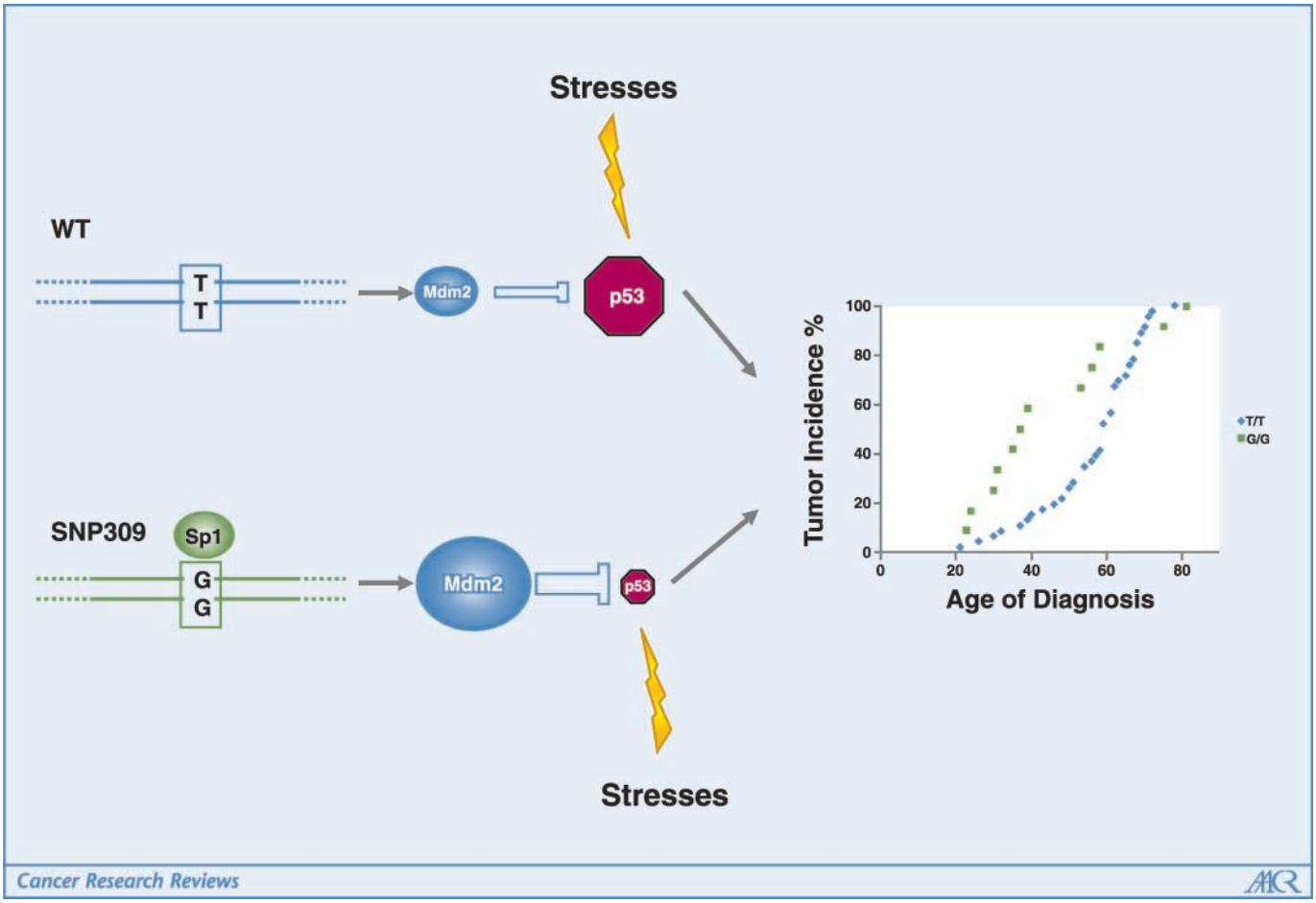

Figure 1. We propose the following model. The G allele of SNP309 raises the basal level of MDM2 in cells. This is due to the creation of an enhanced SP1 transcription factor-binding site in the Mdm2 promoter. The higher basal levels of MDM2 in cells attenuate the p53 apoptotic responses that occur in people in response to DNA damage and other environmental insults. Thus, in some individuals (with a G/G genotype at SNP309), the percentage of cells undergoing apoptosis or cell cycle arrest in response to genotoxic stress is low, and the propagation of cells with mutations, over a life time, permits cancers to arise at younger ages. The mutation rate may be the same in G/G (*green*) and T/T (*blue*) individuals, but the fixation of mutations in clones of cells may be higher in people who are G/G at the *SNP309* locus.

was 27 years and for the T/G and G/G genotypes it was 20 years, whereas the median ages of onset were 27 and 18, respectively (P = 0.031; Table 1). For soft tissue sarcomas, the median age of onset for individuals with a T/T genotype was 14 years of age, whereas for those with a G/T and G/G genotype it was 2 years (P = 0.019). For those who developed breast cancer, the median age of onset was 39 for the T/T genotype and 29 for the G/T and G/G genotypes (P = 0.01; Table 1). Thus, individuals with the Li-Fraumini syndrome with low wild-type p53 activity and with SNP309 (G/G) developed cancers some 10 to 12 years earlier than those individuals with a T/T genotype at that locus. In addition, the SNP309 status also correlated with the number of independent primary tumors that were developed by soft tissue sarcoma patients. Nine patients who were T/T at the *SNP309* locus developed one cancer and two of those nine acquired a second independent tumor. Nine patients who were G/T at the SNP309 locus developed a first cancer, seven of whom acquired a second cancer, four of these patients developed a third independent cancer and one of these individuals acquired a fourth and a fifth cancer. Of the two patients who were G/G at the *SNP309* locus, one developed three independent tumors, whereas the other had five cancers at the time of this study (P = 0.0014; Table 1).

The studies described above surely derive from the interactions of a mutation in one allele of the *p53* gene, which attenuates the p53 response, with SNP309 that also attenuates the p53 response. To examine if SNP309 has a phenotype in people who have no known genetic predisposition for cancer, we collaborated with Frank Bartel, Helge Taubert, and Peter Wuerl to analyze 105 adult sporadic soft tissue sarcomas for their genotype at the *SNP309* locus. Those patients who had a G/G genotype had an average age of onset of 45 years, whereas those individuals with a T/T genotype had an average age of onset of 57 years. The median age of cancer onset for G/G was 38 years and for T/T it was 59 years (P = 0.01). The SNP309 G allele frequency was 50% for patients who acquired their sarcoma before 41 years of age, whereas the G allele frequency in the total population of 105 patients was 33% (P = 0.026). Other than an earlier age of tumor onset, there seemed no other clinical phenotype, which significantly associated with the presence of SNP309 (tumor subtype, stage of tumor, or survival). Like the Li-Fraumeni patients, the distribution of G/G, G/T, and T/T alleles in this cancer cohort was similar to the allele frequencies obtained

[1] Hu et al., unpublished observations.

from a healthy volunteers (not selected for cancer) indicating that in this case, the G/G allele at SNP309 is not enriched in the cancer population. This independent data set of clinical samples reproduces the observations made with the Li-Fraumeni data set so that these correlations between SNP309 and the earlier onset of cancer in individuals seem quite firm.

We interpret these results in the following way (Fig. 1). The G allele of SNP309 raises the basal level of MDM2 in cells. This is due to the creation of an enhanced SP1 transcription factor binding site at that locus. Because SP1 levels and activities are not equally high in all cell and tissue types in humans, there may well be a tissue-specific effect of a SNP that is inherited and in all cells.[1] Thus, one cannot predict which cell or tissue types might be differentially affected by SNP309. The higher basal levels of MDM2 in cells attenuate the p53 apoptotic responses that occur in people in response to DNA damage and other environmental insults. Thus, in some individuals (with a G/G genotype at SNP309), the percentage of cells undergoing apoptosis or cell cycle arrest in response to genotoxic stress is low and the propagation of cells with mutations, over a life time, permits cancers to arise at younger ages. The mutation rate may be the same in G/G and T/T individuals, but the fixation of mutations in clones of cells may be higher in people who are G/G at the SNP309 locus. Whereas this is a reasonable explanation, it is important to appreciate the limitations of studying just one SNP in a pathway. The first indication that other SNPs and genes are contributing to these results is in the comparisons of the average age of cancer onset with the median age of cancer onset provided in Table 1. For example, with the sporadic sarcomas, the difference in the G/G and T/T populations for the average age of onset was 12 years and the difference in the median age of onset is 21 years. This comes about when there are outliers in the data set or individuals who are G/G but develop cancer at an old age comparable with T/T individuals. A reasonable explanation for these results is the fact that other genes and SNPs can modify the G/G MDM2 phenotype and enhance p53 function in a cell. This delays the onset of cancer in an individual with a G/G genotype. The presence of second site modifiers is to be expected; therefore, one SNP cannot be the entire story or explanation. Even so, SNP309 seems to have enough of a robust or determinative phenotype to affect the age of onset of a cancer, on average. The second problem that needs an explanation is how a SNP can decrease the age of onset of a cancer but not the frequency of individuals who get cancer in a population? One possible problem with this question is that the two cohorts employed in this study are small sizes; 66 and 105 individuals with cancer. Larger numbers may be needed to detect a small increase in cancer frequency between a case and control study.

It should be noted that all of the clinical conclusions that come from the Bond et al. study are based upon comparing the ages of onset or number of independent tumors occurring in the cancer cohort (Table 1) and no comparison to a control group needs to be made. There are several explanations why individuals with a particular allele frequency develop cancer at an early age and individuals with the alternate allele frequencies obtain cancers at a later age. The age-specific incidence of cancers seem dependent upon at least three factors: (a) the number of rate limiting mutations required for a given cancer; (b) the mutation rate per cell division; and (c) the net proliferation rate of the affected cells, which is the cell division rate minus the cell death rate (22). The efficiency of the p53 pathway can affect each of these three variables which can lead to both the initiation and the propagation of a cancer. It is perhaps not surprising then that a SNP that can modulate the efficiency of the p53 pathway has a phenotype that affects the age at which individuals develop cancers.

Additional studies with this and other SNPs in the p53 pathway will determine the functions of the *p53* gene and its pathway in the origins of cancer in humans as well as responses to treatment of cancers. There is some hope that understanding a collection of SNPs in a patient may some day may help in the prevention and the treatment of this disease. For example, it may permit a better selection of agents for chemotherapy and predictions for the outcome of therapy. Research over the next few years will likely uncover these SNPs and other variables that will make treatment more informative and more successful.

Acknowledgments

Received 3/22/2005; revised 5/16/2005; accepted 5/17/2005.
Grant support: National Cancer Institute grants PO1 CA 87497 and PO1 CA 65930-08.

References

1. Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. Science 2001;291:1304–51.
2. Sachidanandam R, Weissman D, Schmidt SC, et al. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. Nature 2001;409:928–33.
3. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000;408:307–10.
4. Appella E, Anderson CW. Post-translational modifications and activation of p53 by genotoxic stresses. Eur J Biochem 2001;268:2764–72.
5. Michael D, Oren M. The p53-Mdm2 module and the ubiquitin system. Semin Cancer Biol 2003;13:49–58.
6. Hoh J, Jin S, Parrado T, Edington J, Levine AJ, Ott J. The p53MH algorithm and its application in detecting p53-responsive genes. Proc Natl Acad Sci U S A 2002;99:8467–72.
7. Beckman G, Birgander R, Sjalander A, et al. Is p53 polymorphism maintained by natural selection? Hum Hered 1994;44:266–70.
8. Marin MC, Jost CA, Brooks LA, et al. A common polymorphism acts as an intragenic modifier of mutant p53 behaviour. Nat Genet 2000;25:47–54.
9. Dumont P, Leu JI, Della Pietra AC III, George DL, Murphy M. The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. Nat Genet 2003;33:357–65.
10. Jin S, Levine AJ. The p53 functional circuit. J Cell Sci 2001;114:4139–40.
11. Bond GL, Hu W, Levine AJ. MDM2 is a central node in the p53 pathway: 12 years and counting. Curr Cancer Drug Targets 2005;5:3–8.
12. Mendrysa SM, McElwee MK, Michalowski J, O'Leary KA, Young KM, Perry ME. mdm2 is critical for inhibition of p53 during lymphopoiesis and the response to ionizing irradiation. Mol Cell Biol 2003;23:462–72.
13. Alt JR, Greiner TC, Cleveland JL, Eischen CM. Mdm2 haplo-insufficiency profoundly inhibits Myc-induced lymphomagenesis. EMBO J 2003;22:1442–50.
14. Jones SN, Hancock AR, Vogel H, Donehower LA, Bradley A. Overexpression of Mdm2 in mice reveals a p53-independent role for Mdm2 in tumorigenesis. Proc Natl Acad Sci U S A 1998;95:15608–12.
15. Cordon-Cardo C, Latres E, Drobnjak M, et al. Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. Cancer Res 1994;54:794–9.
16. Leach FS, Tokino T, Meltzer P, et al. p53 Mutation and MDM2 amplification in human soft tissue sarcomas. Cancer Res 1993;53:2231–4.
17. Oliner JD, Kinzler KW, Meltzer PS, George DL, Vogelstein B. Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature 1992;358:80–3.
18. Momand J, Jung D, Wilczynski S, Niland J. The MDM2 gene amplification database. Nucleic Acids Res 1998;26:3453–9.
19. Zauberman A, Flusberg D, Haupt Y, Barak Y, Oren M. A functional p53-responsive intronic promoter is contained within the human mdm2 gene. Nucleic Acids Res 1995;23:2584–92.
20. Ries S, Biederer C, Woods D, et al. Opposing effects of Ras on p53: transcriptional activation of mdm2 and induction of p19ARF. Cell 2000;103:321–30.
21. Bond GL, Hu W, Bond EE, et al. A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. Cell 2004;119:591–602.
22. Knudson AG. Two genetic hits (more or less) to cancer. Nat Rev Cancer 2001;1:157–62.