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Research Focus

When mutant p53 plays hide and seek: a new challenge for diagnosis and therapy?

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***p53* missense mutations observed in human cancers are often associated with an increased level of p53 protein in the tumour. Using mouse models, Terzian *et al.* recently showed that this accumulation of mutant p53 protein is not associated with specific properties of the protein itself but instead depends on the endogenous genetic background of the tumours and on two important genes, *mouse double minute 2* (*Mdm2*) and the cyclin kinase inhibitor *p16^{INK4a}*. Mice expressing mutant p53 in the absence of Mdm2 display more aggressive metastatic tumours. In light of these observations, targeting the MDM2–p53 interaction for therapy of human cancer could be more complicated than previously anticipated.**

p53 regulation

The tumour suppressor p53 acts as a stress-activated protein after DNA damage, oncogene activation, hypoxia or metabolic alterations [1,2]. p53 acts primarily as a nuclear transcription factor that regulates hundreds of genes associated with cell-cycle arrest, apoptosis and senescence [3]. More recent studies indicate that cytoplasmic p53 can also regulate apoptosis and autophagy through transcriptionally independent mechanisms [4,5]. p53 regulation is predominantly carried out at the protein level through an auto-regulatory feedback loop with the mouse double minute 2 (MDM2) protein. In a normal, unstressed cell, p53 activates the transcription of Mdm2, which in turn binds tightly to p53 and targets it for proteasomal degradation (Figure 1a). Upon cellular stress, Mdm2 binding to p53 is impaired via different mechanisms, leading to the accumulation of p53 protein, which in turn, through various post-translational modifications such as phophorylation and acetylation, becomes transcriptionally competent (Figure 1b).

Mutant p53 in human cancer

Alteration of p53 pathways is one of the major hallmarks of human cancer and occurs via multiple mechanisms, including missense mutations in the p53 gene, MDM2 accumulation, deletion of the CDKN2A gene encoding p16^{ARF} or nuclear exclusion of the p53 protein (Figure 1b) [6]. p53 missense mutations, which are found in a significant proportion of human cancers (40–50%), are often associated with p53 protein accumulation in the nucleus of tumour cells (Figure 2a). Immunohistochemistry (IHC) has been widely used to assess p53 status in human tumours. Despite numerous, sometimes controversial studies pointing out the limitations of this approach, IHC is still commonly used [7].

The mechanism leading to mutant p53 accumulation in the nucleus has long eluded cancer biologists. Initial *in vitro* studies suggested that mutant p53 was more stable than the wild-type protein, either due to an internal change of conformation or through binding to cellular proteins, such as the chaperone heat shock protein 70 (HSP70). However, these hypotheses were not supported by the clinical observations of patients with Li-Fraumeni syndrome (LFS), a hereditary cancer predisposition syndrome associated with increased risk of developing cancer. The most common types of cancer found in families with LFS include osteosarcoma (bone cancer), soft tissue sarcoma, leukaemia, breast cancer, brain cancer and adrenal cortical tumours [8]. Approximately 70% of families with LFS will have a heterozygote germline mutation in the p53 gene and will express both wild-type and mutant p53 in their normal cells. Despite this genotype, in these patients, accumulation of mutant p53 protein can only be visualized in tumours, whether or not the wild-type *p53* allele is retained. This finding suggested that the tumour environment is an important component for stabilization of the mutant p53 protein. Accumulation of mutant p53 protein might have unfavourable consequences because some mutant p53 proteins seem to have a gain-of-function activity. The acquired function occurs either through activation of a specific transcription programme or owing to a dominant negative interaction with other proteins [9,10]. Although wild-type p53 is considered to be a tumour suppressor gene, it is now well established that mutant p53 can behave like a dominant oncogene that can contribute to neoplastic progression.

Human and mouse tumours express high levels of p53

The first mouse model developed to investigate the function of p53 comprised classical knockout (KO) mice lacking the entire *p53* gene [11]. These mice were viable but prone to cancer. However, they do not reflect the situation of human tumours that carry p53 missense mutations, and this model is thus inadequate for assessing the consequences of the oncogenic activation of mutant p53. Therefore, second-generation mouse models were developed with knock-in mutations in *p53*, similar to the mutations found in human cancer [12,13]. These mice are viable and display a high frequency of carcinoma and metastasis, a feature not found in KO mice, which display predominantly T-cell lymphomas. The spectrum of

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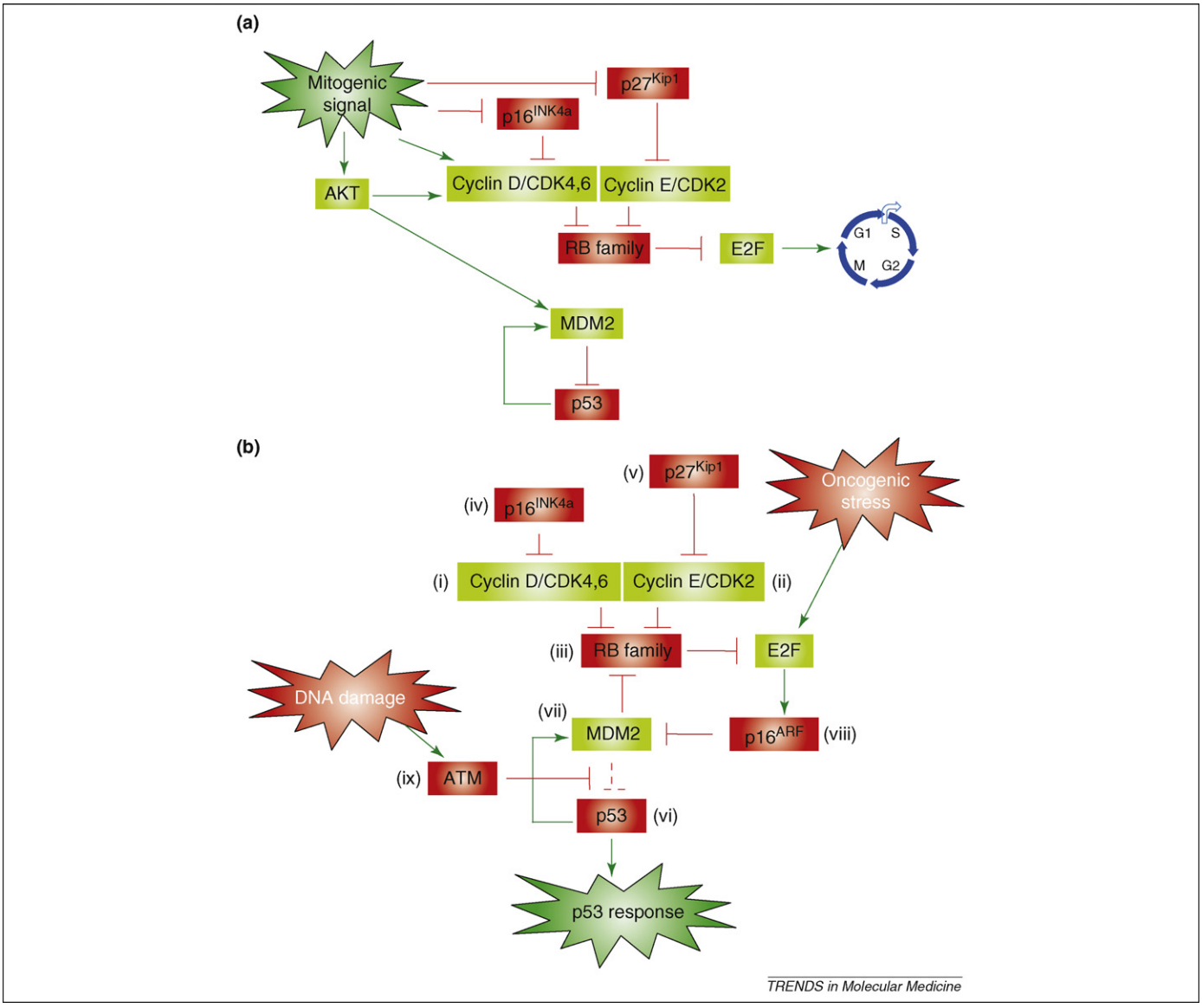

Figure 1. Regulation of the p53–mdm2–Ink4A pathway. One of the most important features of cell-cycle regulation is the discrimination between normal harmful physiological mitogenic signals and abnormal oncogenic proliferative signals or responses to cellular stress. **(a)** In a normal cell, the G1–S transition is regulated by the retinoblastoma (RB)-family proteins, which sequester and inhibit the E2F transcription factors. Upon a normal mitogenic signal, the various cyclin-dependant kinase (CDK)–cyclin complexes will phoshorylate RB proteins, releasing the E2F transcription factors that will activate the expression programme needed for the S phase. This transition can be negatively regulated by the cyclin-dependant kinase inhibitor (CDKI), $p16^{INK4a}$ and $p27^{KIP1}$. During this cell-cycle progression, the level of p53 protein is kept low because it is targeted by MDM2 for proteosomal degradation. The AKT protein, activated by these mitogenic signals, activates MDM2 to keep p53 to a minimum level upon this normal cell response. **(b)** After DNA damage or oncogenic stress, the p53–MDM2 loop is impaired, allowing p53 activation and an adequate p53 response. Oncogenic activation leads to the inactivation of RB proteins and a massive release of E2F transcription factors, which activate the transcription of the CDKN2A gene that codes for both $p16^{ARF}$ and $p16^{INK4A}$. $p16^{ARF}$ binds and sequesters MDM2 in the nucleoli and thereby stabilizes p53 in the nucleoplasm. DNA-damage activation of p53 occurs through phosphorylation of p53 and MDM2 by several kinases, including the ataxia telangectasia mutated (ATM) protein, which impairs the association between the two proteins and releases active p53. In human cancer, this pathway is the target of numerous abnormalities, and all of these genes can be considered as either oncogenes (green) or tumour-suppressor genes (red): (i) amplification of *cyclin D1* gene (breast cancer); (ii) accumulation of CYCLIN E protein (breast cancer); (iii) inactivating mutations of or deletion of the RB gene (retinoblastoma, lung cancer); (iv) deletion or promoter inactivation via methylation of CDKN2A that encodes $p16^{INK4a}$ (numerous types of cancer); (v) decrease in expression of $p27^{Kip1}$; (vi) inactivating point mutation of the p53 gene (numerous types of cancer); (vii) amplification of the MDM2 gene (sarcoma); (viii) deletion or mutation of the CDKN2A locus coding for $p16^{ARF}$ (numerous types of cancer); (ix) inactivating mutation of the ATM gene (B-cell lymphoma).

tumours and metastasis was different among the different mutant p53 proteins studied in these mice or depending on the genetic background of the mice; this spectrum more closely resembled human tumours.

Terzian *et al.* used this knock-in mouse model to analyse the expression of mutant p53 in various tissues [14]. Their first observation was that mouse tumours behaved more like human tumours; they displayed mutant p53 accumulation in the nucleus, whereas in normal cells mutant p53 was undetectable (Figure 2a). This feature was observed in both heterozygote (one mutant and one wild-type allele) and homozygote (two mutant alleles) animals. This is an important finding because it rules out any function of wild-type p53 in regulating the expression of mutant p53 in normal cells. Another crucial observation was that only 70% of these tumours displayed p53 accumulation in homozygote animals.

Both p53-positive and -negative tumours could be found simultaneously in animals with several independent primary neoplasms, indicating that additional modifications are necessary to display this phenotype [14]. This is in line with observations from clinical studies that 10–

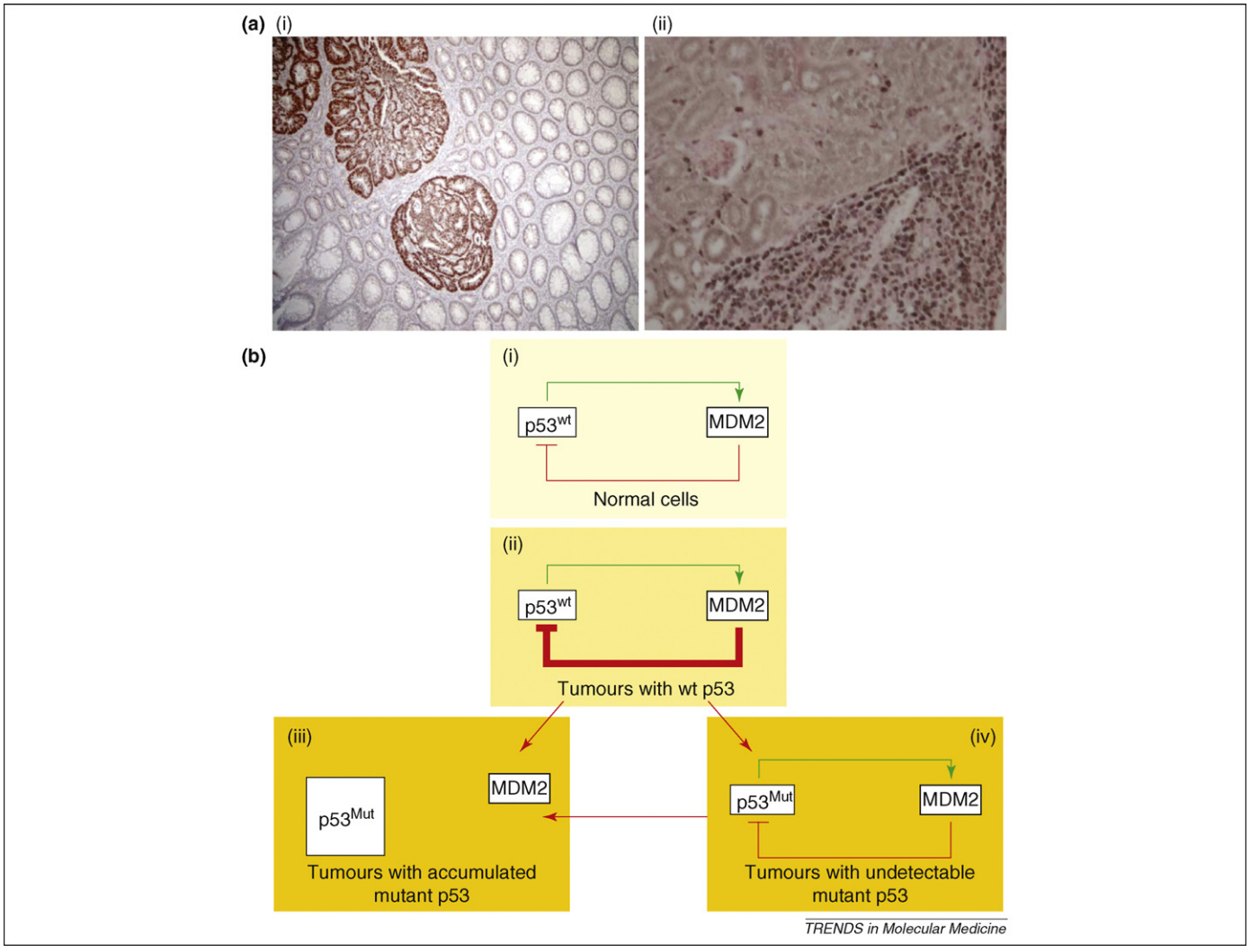

Figure 2. p53 and MDM2 in cancer cells. **(a)** p53 staining in tumours: (i) a human colorectal carcinoma (photo courtesy of P. Hall) and (ii) a mouse tumour (photo courtesy of G Lozano and Y. Su), both expressing a mutant p53 in tumour cells, were stained using a p53 monoclonal antibody. Normal cells are not stained. **(b)** The p53–MDM2 loop in normal and tumour cells in a normal environment. (i) In normal cells, the level of p53 is restrained by MDM2 as long as no stress impairs this regulation and activates p53 growth-arrest and apoptosis activity. Whether or not this low level of p53 also contributes to genetic stability is still an open question. Several possibilities can be observed in tumour cells. (ii) In the absence of p53 mutations, the p53 pathway can be impaired by an abnormal level of MDM2. (iii) In tumours with high levels of p53 caused by loss of regulation by MDM2 and $p16^{INK4a}$, the accumulation of mutant p53 is unregulated. (iv) In tumours that express low levels of mutant p53, Terzian *et al.* showed that regulation of p53 accumulation is still intact; however, they also observed selection for cells with high levels of mutant p53. Whether this selection for metastatic tumours that express high levels of mutant p53 occurs during natural tumour progression or after specific treatment needs to be carefully investigated. Abbreviations: Mut, mutant; wt, wild type.

30% of human tumours with *p53* missense mutations do not express p53 protein [15,16]. Therefore, this study further emphasizes the point that IHC is not sufficient to accurately assess p53 status in human tumours, because this accumulation is not a direct property of the p53 protein but is also related to the genotype of the tumour. Also, identification of *p53* mutations in human tumours by sequencing is necessary because there are several classes of mutant p53 with different characteristics and heterogeneous clinical phenotypes [17]. With recent progress in sequencing technology (higher throughput and sensitivity, lower cost), molecular analysis should become the gold standard for assessing p53 status clinically.

MDM2 and $p16^{ARF}$ are important regulators of mutant p53 accumulation

One of the main regulators of p53 protein stability is MDM2, an E3 ubiquitin ligase that targets p53 for degradation in the proteasome. Because MDM2 expression is under positive control by p53, this auto-regulatory loop in normal cells allows very tight regulation of p53 expression (Figure 1a). The importance of this interaction is emphasized by the observation that mice lacking both *Mdm2* alleles die *in utero* at an early stage, whereas this phenotype is reversed when these mice also lack p53 [18]. Mice lacking both p53 and Mdm2 are viable but prone to cancer. Besides MDM2, other proteins such as COP1 (constitutively photomorphogenic 1), PIRH2 (p53 induced protein with a RING F2 domain) or MDM4 also regulate p53 stability. MDM4 is a structural homologue of MDM2 that also binds and inhibits p53.

To investigate whether Mdm2 regulates the level of mutant p53 in normal cells, Terzian *et al.* analysed novel mouse models in which the Mdm2 gene was deleted. Analysis of the p53 mutant knock-in mice in an *Mdm2*-null background led to striking results [14]. First, absence of Mdm2 led to an accumulation of p53 protein in both normal and tumour cells (except in liver). Second, these mice died from cancer significantly earlier than mice expressing only mutant p53 and wild-type Mdm2. Third,

~17% of these mice developed metastatic tumours, a phenotype not displayed when the *Mdm2* gene was intact.

It is unknown how loss of p53 regulation by Mdm2 can increase the metastatic phenotype. The most obvious hypothesis for explaining this phenotype is that mutant p53 accumulation in normal cells changes the timeline for alteration of the p53 pathway, for example, an earlier gain-of-function activity of mutant p53 could lead to a metastatic phenotype (Figure 2b). This might also explain the accelerated tumourigenesis observed in the absence of Mdm2 [14].

Further analysis of the p53 pathway also indicates that inactivation of the retinoblastoma (RB) pathway, either via $p16^{INK4A}$ deletion or through cyclin D accumulation, is an important requirement for the specific accumulation of mutant p53 in the tumours [14].

Therapeutic implications

p53 is an attractive target for the development of therapeutic drugs [19,20]. In tumours expressing mutant p53, small molecules that can rescue p53 function have been developed. In tumours with wild-type p53, inhibition of MDM2 has been proposed because it will lead to the induction of p53 activity. Small compounds binding either to p53 or to MDM2/Mdm2 have been isolated and have been shown to induce a p53-dependent apoptosis in tumour cell lines or in mice bearing tumour grafts [19,20]. Although such drugs targeting the interaction of p53 and MDM2 could be very efficient for short-term treatment, longer exposure to the drugs could have adverse effects, as suggested by the work of Terzian *et al.* [14]. Indeed, it is possible that targeted treatment of tumours initially expressing wild-type p53 could lead to resistance mechanisms owing to the selection of cells that express high levels of mutant p53 and are unregulated by MDM2; these cells would therefore have a more aggressive metastatic phenotype. Similar mechanisms are commonly observed for other drugs, such as the two kinase inhibitors Gleevec® or Iressa®, and selection of mutant p53 during tumour development or chemotherapy has been observed in both humans and mice [21]. The mutant p53 selected could either be pre-existing in very few cells before treatment or could occur during treatment.

Concluding remarks

Terzian and colleagues' work with mouse models has confirmed several clinical observations. (i) p53 accumulation in tumour cells is not an intrinsic property of the p53 protein but is associated with the genotype of the tumour. (ii) p53 mutations are not always associated with p53 accumulation. (iii) Downregulation of mutant p53 in normal cells by wild-type p53 is an important control that prevents the occurrence of aggressive and metastatic tumours. The work by Terzian *et al.* was performed with mutant p53 that contains a mutation corresponding to the human hot-spot mutation R175H, a mutant that displays a profound change of conformation and is associated with multiple gain-of-function activities. It will be important to investigate whether other types of regulation of other p53 mutants by MDM2 lead to the same phenotype (Box 1).

Beyond the classical binary view of tumours with either wild-type or mutant p53, it remains to be defined whether tumours that display undetectable levels of mutant p53 controlled by MDM2 could be a novel entity, in which mutant p53 behaves like a time-bomb waiting for release.

Box 1. Outstanding questions

- Does mutant p53 expressed in normal cells contribute to neoplastic progression and, if so, how does this occur?
- How does loss of the RB pathway lead to p53 accumulation?
- How does the absence of Mdm2/MDM2 lead to metastatic tumours?
- What is the status of MDM2 in highly metastatic human tumours?
- What are the exact roles of the other ubiquitin ligases that target p53 (COP1 or PIRH2)?
- What is the function of MDM4 in human cancer?

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