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p53: a molecular marker for the detection of cancer

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Abstract

Background—The p53 gene is the most frequently mutated gene in cancer and accordingly has been the subject of intensive investigation for almost 30 years. Loss of p53 function due to mutations has been unequivocally demonstrated to promote cancer in both humans and in model systems. As a consequence, there exists an enormous body of information regarding the function of normal p53 in biology and the pathobiological consequences of p53 mutation. It has long been recognised that analysis of p53 has considerable potential as a tool for use in both diagnostic and, to a greater extent, prognostic settings and some significant progress has been made in both of these arenas.

Objective—To provide an overview of the biology of p53, particularly in the context of uses of p53 as a diagnostic tool.

Methods—A literature review focused upon the methods and uses of p53 analysis in the diagnosis of sporadic cancers, rare genetic disorders and in detection of residual disease.

Conclusion—p53 is currently an essential diagnostic for the rare inherited cancer prone syndrome (Li-Fraumeni) and is an important diagnostic in only a limited number of settings in sporadic disease. Research in specific cancers indicates that the uses of increasingly well informed p53 mutational analysis are likely to expand to other cancers.

Keywords

cancer; diagnosis; immunohistochemistry; Li-fraumeni syndrome; p53; tumour suppressor gene

1. Introduction

What is there to say about p53 that we don't already know? A recent search showed that there were more than 45,000 papers on p53 in PubMed, and this represents roughly 2% of

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all papers published on cancer. Given that we have so much information, is there any need for yet another review? The answer in this instance perhaps lies in the relative paucity of information regarding the use of p53 as a diagnostic tool. Since its discovery in 1979 [1-3] the study of this 53 kDa protein and its activities has been at the forefront of much of the activity in cancer research. When first discovered, p53 was identified as a protein target for a viral oncogene and was later shown to possess intrinsic transforming activity [4]. It gradually became clear that this transforming potential derived from mutations in the p53 gene and 10 years after its initial discovery it was found that wild-type p53 rather acts to suppress tumourigenesis [5-7]. Although p53 has been linked with a number of cellular processes, its connection with cancer has always remained an extremely close one. The realisation that the p53 gene is frequently mutated in human cancer [8,9] led to hopes that it may become diagnostically useful and moreover it may become the target of the much hoped for magic bullet of cancer therapy [10-12]. That this has not happened is to a great extent due to the underlying biology of p53. This review therefore concentrates first on the biology of p53 and then discusses some attempts to use p53 as a diagnostic tool, and finally ends with some observations on what could be done to advance the present situation.

2. p53

2.1 The biology of p53 (with an emphasis on elements of relevance to the use of p53 for diagnosis)

As mentioned above, p53 was first identified in a tumour cell line as a 53 kDa protein that co-purified with the oncogenic large T antigen of SV40 following immuno-purification from SV40-transformed mouse cell extracts using antibodies that had been raised against large T antigen [2,13]. This protein possessed characteristic oncogenic properties, for example, expression of the cDNA transformed and immortalised cells in culture [7,14,15]. Later, it became clear that not all p53 cDNA clones cooperated with activated RAS to transform cells and eventually it was found that many of the earlier cDNAs harboured mutations [16]. Subsequent studies showed that wild-type p53 could suppress cell proliferation and that this protein bound to DNA with a preference for a consensus sequence [6,7,17]. We now know that one of the most important activities of p53 is as a DNA sequence specific transcription factor [18,19]. The activities of p53 have been extensively reviewed, see for example [20]. p53 plays a vital role in coordinating responses to a broad range of (predominantly but not exclusively) genotoxic cellular stresses (see Figure 1) resulting in a variety of distinct outcomes that include cell cycle arrest, DNA damage repair, senescence and apoptosis [21]. Stress is detected by a range of enzymes that signal to p53 through promoting post-translational modifications such as phosphorylation, acetylation, methylation and SUMOylation [22,23]. Although it is not completely understood how this occurs and how multiple modifications are coordinated, p53 modifications result in both upregulation of the p53 protein, in most instances, and stimulation of p53 transcriptional activation. Activation results in increased transcription due to p53 binding to a number of different promoter regions (some estimates suggest that this number is as high as ~ 1600 [24], although many estimates are considerably lower [25] and the number of functionally validated direct transcriptional targets is considerably less).

Interestingly, there is no gene for which it has been proven that its transcriptional upregulation by p53 is essential (i.e., no essential gene has yet been identified that has been shown to depend for its essential function upon upregulation by p53, not even MDM2). One gene that could be argued to display this relationship is LIF, a p53 transcriptional target that plays an important role in implantation and thus contributes to fertility. However, LIF is not essential for normal adult development [26]. Since some of the outcomes promoted by p53 are mutually exclusive, one could anticipate that the decision of which outcome will occur would be determined through selective transcriptional activation. It would then follow that the specificity of p53 activation would result from integrating the stress signals that could be achieved via multiple post-translational modifications of the p53 protein altering promoter specificity. This attractive 'p53 smart' model is not universally supported by studies performed to date and thus an alternative 'p53 dumb' model has also been considered [27,28]. In this latter model p53 activates a broadly similar panel of genes regardless of stress and the consequences are determined by other factors, including the extent/duration of the stress signal, the cell type and other signals being received by cells. Regardless of how these decisions about the nature of the response are made, p53 activation must then instigate the specific response. In some cases how the individual p53 responses are mediated is well characterised. For example, the mechanisms through which p53 promotes cell cycle arrest have been well defined. In response to DNA damage (particularly UV irradiation), p53 promotes G1 cell cycle arrest and DNA repair by promoting increased expression of CDKN1A (p21 waf1/cip1) [29,30]. In cycling cells CDKN1A binds to cyclin D and is a necessary component of the active complex of cyclin D with its cognate CDKs (4 and 6) [31]. Following DNA damage, two events occur; firstly, existing cyclin D is degraded [32] and then following transcriptional activation by p53, the levels of CDKN1A increase [33]. The important consequence of both of these events is the production of free CDKN1A protein that can bind to and inhibit the activity of cyclin E/CDK2 and in so doing, halt cell cycle progression by preventing further phosphorylation of pRB [34]. In addition, upregulation of CDKN1A promotes DNA repair probably through binding to PCNA and promoting DNA repair at the expense of DNA replication [35,36]. Similarly well delineated is the pathway connecting p53 activation following DNA damage (particularly ionising radiation) to G2 arrest. A key transcriptional target of p53 in this setting is the 14-3-3 σ protein [37,38] (reviewed in [39]). Following DNA damage induction by ionising radiation, p53 is modified on serines 15 and 20 by ATM [40-43] (studies in mice have confirmed that the equivalent modifications are necessary for some DNA damage responses [44,45]), resulting in activation of p53. 14-3-3 σ is upregulated as part of this stress response [38] and binds to CDC2, sequestering the protein in the cytoplasm and thus preventing cell cycle progression [39]. Other parts of the p53 response are less well understood. For example, in response to oncogenic activation and also in cells exposed to ionising radiation, p53 activation can efficiently promote cellular senescence through mechanisms that remain to be defined (see [46,47] for review).

The apparently myriad mechanisms through which p53 induces apoptosis are also poorly understood. That p53 is a potent inducer of apoptosis has been undisputed for some time [48]; indeed this pathway is thought by many to be the most important for mediating p53 tumour suppression, although there is also good evidence to the contrary [49]. p53 can elicit

an apoptotic response through multiple pathways and can act upon both the intrinsic and extrinsic apoptotic machinery through both p53 transcriptional activation dependent and independent mechanisms [50]. This apparent redundancy may have evolved to ensure that apoptosis is effectively activated as a failsafe response to genotoxic or oncogenic stress. In addition to acting as a transcriptional regulator of pro-apoptotic genes (for example p53 induces expression of the BH3 protein Bax), p53 also acts as a transcriptional repressor of antiapoptotic genes such as Bcl-2 [51,52]. Furthermore, through direct protein–protein interactions p53 can bind to apoptotic regulatory proteins and stimulate apoptosis [50].

Regarding general p53 biology, it is important to appreciate that for all of the critical functions regulated by p53 that are central to the prevention of cancer in higher organisms, p53 is not an essential gene [53]. Mice lacking the ability to produce the full-length protein or possessing missense mutations in coding exons of the p53 gene grow to adulthood, although they invariably develop a wide spectrum of cancers and die prematurely as a consequence. It should be noted, however, that there is evidence in some of these studies of a role for p53 in normal development. Several studies of p53 knockout mice have noted a bias against females with homozygous disruption of the p53 gene [54]. These studies have described the occurrence of exencephaly in female embryos and this suggests that loss of p53 leads to abnormalities in the development of these animals. The reason for this is not clear, although it has been suggested that it may be due to differences in diet in addition to genetic background [55].

To understand the problems facing those wanting to use p53 as a diagnostic tool in cancer, one of the key features of its biology that needs to be understood is how it is regulated in normal cells and how this breaks down in cancer cells. As can be seen above, many different cellular stresses converge on p53 and the outcomes of these may be irreversible. Clearly, therefore, it is vitally important to the organism to regulate p53 activity effectively to prevent inappropriate activation with potentially lethal consequences, while still retaining the ability to activate critical tumour suppressive functions. That the consequences of inappropriate activation can be quite literally lethal is apparent from studies of mice lacking either of two essential p53 negative regulatory genes: MDM2 and MDMX (MDM4). Deletion of either of these genes results in an early embryonic lethality in the absence of exogenous stress and the fact that this is rescued by concomitant deletion of p53 demonstrates that the lethality observed is due to p53 [56–58]. The interaction between p53 and MDM2 is of particular relevance to cancer, since a breakdown in this interaction results in one of the key phenotypic features of cancer cells, namely, upregulation of p53 protein [59]. In normal cells p53 is maintained at a low level by the action of the MDM2 proto-oncogene [60]. The MDM2 protein interacts directly with p53 and indeed most of the oncogenic effects of MDM2 are due to its ability to negatively regulate the p53 tumour suppressor protein [61]. p53 levels and activity are regulated by MDM2 and it is increasingly clear that the regulation of p53 levels is substantially due to the ability of MDM2 to act as a ubiquitin ligase with specificity for, *inter alia*, p53 [62–65]. MDM2 targets p53 for ubiquitylation, leading to export of p53 from the nucleus and/or degradation by proteasomes [66,67]. Of critical importance, MDM2 is also a transcriptional target of p53 and thus an autoregulatory feedback loops exists between these two molecules [59]. Following stress, the interaction between p53 and MDM2 is prevented through post-translational modifications of p53 and

perhaps also of MDM2, resulting in a rise in the level of p53 protein [68]. As a consequence, p53 is also activated (see [69] for a review) and one of the target genes that is upregulated is MDM2. Whether the MDM2-protective post-translational modifications of activated p53 are then removed to permit reassociation and thence degradation, or whether the *de novo* synthesised protein lacking the modifications gradually becomes the majority of the p53 in the cell through gradual turnover of the modified stabilised p53 is unclear. By whatever means this occurs, the level of p53 in the cell will return to the normal low homeostatic level following removal of the stress stimulus, and this is dependent upon MDM2 [70]. In cancer cells, high levels of p53 protein are frequently detected and a considerable number of studies have attempted to use this information to predict a range of patient outcomes and responses (discussed in [71]).

It has been suggested that the reason that mutant p53 is upregulated in cancer is due to failure of mutant p53 to transactivate expression of MDM2 and thus there is a breakdown in the autoregulatory feedback loop [72]. In cancers where high levels of mutant p53 arise in the absence of raised levels of MDM2, this may well be the correct explanation for the phenotype. There are however many instances where high levels of both MDM2 and p53 occur, and these cases are not explained by this model. This phenotype/genotype correlation (high MDM2 expression with mutant p53 expression) has been recorded in some common cancers such as bladder, and has been associated with increased relative risk of death [73]. Elsewhere, in renal cancer for instance, there is a significant association between raised p53 levels and upregulation of MDM2 ($p < 0.00004$, p53 mutational status not defined) and moreover of this phenotype is linked with poor outcome ($p < 0.00179$) [74]. This combination of high levels of both proteins is not confined solely to the kidney, having also been observed in prostate, bladder and oesophageal cancers [73-76]. In these cases, the reasons for p53 upregulation are unclear. A mechanism must exist that prevents degradation of the p53 protein by MDM2. A number of possible explanations are available, although none are completely satisfactory, as Figure 2 shows.

High levels of both proteins could reflect constitutive post-translational modification (e.g., by phosphorylation) of wild-type p53. This might occur as part of the stress response to oncogenic signalling and would protect p53 from MDM2-mediated degradation through constantly stimulating post-translational modification by pathways generally used to activate wild-type p53 in normal cells exposed to stress. This would also generate higher levels of MDM2 expression that would be driven by p53. This is certainly not the case in bladder cancer, for which mutations of p53 exist side by side with upregulation of MDM2 (associated with reduced survival), and thus still begs the question why is MDM2 upregulated in these cancers? Gene amplification cannot provide the explanation for this as it has not been documented in this cancer. Other possible explanations include increased expression of MDMX, which would result in inhibition of p53 without degradation, although again this would not account for the increased MDM2 expression. Alternatively, upregulation of p14^{ARF}, which possesses both p53-dependent and independent tumour suppressor activity. The latter, which is achieved through its ability to activate p53 via binding to and inhibiting MDM2, could lead to increased p53 and MDM2 proteins. However, such upregulation seems unlikely, since loss of p14^{ARF} expression is more typically observed in tumours, and given its tumour suppressive function, this is hardly

surprising [77]. Another candidate might be a chaperone such as Hsp90, which might bind to either or both proteins preventing degradation of p53 [78]. Clearly more work will be required to determine the basis for this apparently significant phenotype/outcome correlation.

2.2 p53 as a diagnostic tool

Since the discovery that p53 protein is upregulated in many cancers, there has been the attractive possibility that detection of this upregulation might provide the basis for a diagnostic assay [79]. There are several problems with this approach. Firstly, p53 is upregulated normally in cells exposed to a range of cellular stresses. The first example of this for normal skin was documented following exposure to UV irradiation [80]. As described above, p53 upregulation results from stress signalling to both p53 and MDM2, leading to the escape of p53 from MDM2-mediated ubiquitylation and consequent degradation by proteasomes. This contributes to confounding the interpretation of raised p53 protein levels as a surrogate indicator of mutations, albeit that in tumours this expression is likely to be more stably elevated than occurs following a normal transient response to stress. Another problem with the use of detection of p53 expression as a diagnostic tool is that mutations arise in p53 in cells that do not go on to develop into tumours. Given all this, combining p53 protein measurements with other techniques can be a highly effective strategy. For example, in a study of p53 mutation in skin the detection of high levels of p53 was made first from biopsies and this was then followed up with direct sequencing of PCR products to confirm the presence of mutations [81]. In the absence of confirmatory analysis by either sequencing or functional assay such as FASAY (functional assay of separated alleles in yeast) [82,83], immunohistochemistry for p53 can only be used to infer p53 status.

The detection of p53 mutations has been employed more extensively and more reliably than examining protein levels in clinical samples. In discussing the use of p53 mutational analysis in diagnosis, a distinction should be made between somatic p53 mutations and germline mutations because there is a critical difference in the consequences of p53 mutation in these two settings which is determined by additional essentially stochastic events. In the case of inherited defects in the p53 gene (or in regulators of p53), there is diagnostic value in detecting these genetic lesions in isolation. Li-Fraumeni disorder is a rare autosomal dominant condition (it is estimated that fewer than 400 families have been identified worldwide) that is identified clinically by a proband with a sarcoma who is under 45 years of age having a first-degree relative under 45 years of age with any cancer and with a third family member, either a first or second-degree relative, under 45 years of age with cancer or with a sarcoma at any age [84]. Individuals meeting these criteria are genetically counselled prior to screening for p53 mutations. This is normally accomplished by direct sequencing of PCR products for either all of the coding exons of p53 (2 – 11 or a subset of these; some services screen 3 – 10 and this identifies > 95% of p53 mutations). Such screening can be used to confirm the diagnosis of Li-Fraumeni and is also useful for identifying carriers of the mutation which can be performed prenatally [85]. Mutation of one allele of the p53 gene has been shown to be the genetic lesion in around a third of the families affected by this syndrome and not all cases of Li-Fraumeni-like syndromes are caused by p53 lesions. For example, some kindreds have been identified that have mutations in the CHEK (CHK) gene

[86]. Nevertheless, inactivating mutations of the TP53 gene have a high degree of penetrance and the detection of these is therefore important diagnostically.

The identity of the specific mutation is also informative. Studies have identified that certain mutations were associated with increased risk of developing certain cancers; for example the IARC p53 database contains data from a large number of families with germline TP53 gene mutations and analysis of this indicates that brain tumours were strongly associated with missense mutations in a region encoding a part of the p53 protein that interacts directly with DNA, whereas adrenal gland carcinomas were linked with mutations elsewhere in the core domain. It is worth pointing out that some germline mutations of p53 may not cause Li-Fraumeni syndrome. In one study of a series of 36 individuals with childhood adrenocortical carcinoma from families with little or no evidence of a cancer syndrome, 35 individuals were found to harbour a germline p53 mutation (R337H) [87]. Similarly other, presumably lower penetrance, p53 mutations were detected in another study of childhood adrenocortical carcinoma [88]. Thus, while most inactivating germline p53 mutations cause Li-Fraumeni syndrome, occasionally p53 mutations may be detected in the germline of individuals that do not have this disorder. Another example of this comes from the description of a series of individuals with breast cancer from families with histories of breast cancer but which lacked mutations in BRCA1 and BRCA2. Three individuals were found to harbour single point mutations in an intron (two heterozygous and one homozygous) in the p53 gene and it was determined subsequently that these families had pedigrees which resembled a late onset form of a Li-Fraumeni-like syndrome [89].

The utility of p53 mutation detection in diagnosis is quite different when considering somatic lesions. Once the importance of the function of p53 as a tumour suppressor was realised in the late 1980s and early 1990s, it was widely anticipated that detecting mutations in p53 would have diagnostic potential and moreover that p53 would become an important drug target [11]. With notable exceptions, the former has yet to become a widely applicable reality, while the latter continues to be an immense challenge. With respect to diagnosis, there are a number of problems. Most obvious is that p53 mutations are detectable in normal tissues by sensitive methods [90]. A range of highly sensitive techniques have been developed for the detection of p53 mutations and continue to be tested [91-93]. However, the very sensitivity of some of these methods may contribute to uncertainty regarding the significance of such detection since a mutation in TP53 in a single cell may not be diagnostic of anything except the presence of the mutation itself [81]. The use of sensitive methods to detect early and therefore presumably relatively low abundance p53 mutations has demonstrated, not surprisingly, that with sensitivity comes increasing detection of spontaneous mutations in otherwise noncancerous tissues. One of the most widely used methods for screening for TP53 mutations is the p53 FASAY and this has been applied to the analysis of many hundreds, if not thousands, of cancer samples [83,94]. The p53 FASAY is conceptually simple: a yeast strain that is auxotrophic for adenine due to a mutation in the *Ade2* gene has an additional copy of the gene under the regulation of a p53-responsive promoter. The assay relies upon the fact that in media with a low concentration of adenine, yeast that contains nonfunctional (i.e., mutant) p53 produces red colonies (due to the accumulation of a red pigmented intermediate of the adenine biosynthetic pathway), whereas yeast with wild-type p53 produces white colonies [95]. One of the limitations of the

method is that only part of exons 4 – 10 are tested by the assay but it is estimated that this encompasses more than 95% of all p53 mutations [96]. One of the strengths of the FASAY is that it can be used to detect functionally significant mutations in biopsies that may also contain normal tissue or even in blood samples from chronic lymphocytic leukaemia patients that may have relatively low tumour burdens. Using this approach, tumour samples that represent as little as 1 – 5% of the total biopsy may be detected (Figure 3). Less than a few hundred nanograms of total cellular RNA can be used successfully to generate a p53 cDNA product for assaying in this system. This makes the FASAY an extremely attractive method for detecting p53 mutations, not only to establish the presence of mutations in actual tumours but also for the detection of mutations relatively early in tumour development.

Using an even more sensitive approach involving PCR from laser captured single cell samples of human skin, it has been revealed that mutations of TP53 are common in normal skin which has been exposed to UV light and moreover that these mutations persist for months after the exposure ceases [81]. Another sensitive approach, albeit one that is of more limited utility due to its dependence upon restriction enzyme sites being contiguous with mutations, has also found that TP53 mutations arise in precancerous or inflammatory and thus potentially normal cells. The restriction site mutation (RSM) assay has been used to detect p53 mutations in gastritis and in premalignant metaplastic tissue from Barrett's oesophagus samples [91,97]. A considerable effort has been made to develop high-throughput mutational analysis for p53 based upon DNA microarray technology. Several different strategies have been adopted but none have reliably demonstrated to be capable of replacing the need for sequencing to confirm mutations. One early approach was the Affymetrix p53 GeneChip™ (the p53 GeneChip is no longer a catalogue product), which used oligonucleotides tiled in groups of five with either G, A, T, C or a single base deletion in each position [98]. Early iterations of the p53 GeneChip™ showed that the technology could be used successfully on clinical material and could potentially detect as little as 5% mutant in the context of wild-type signal. However, several studies concluded that the GeneChip (at least as originally designed) should not be used as a standalone technique, primarily because of limitations in the design of the array, many of which could be resolved by revision of the design, but also due to technical issues relating to sample preparation that compromised the effectiveness of the array [99,100]. Nevertheless, recent large-scale studies based upon this technology have demonstrated the effectiveness of a strategy that combines such oligonucleotide arrays with other high-throughput methods such as denaturing high-performance liquid chromatography (Transgenomic Surveyor® DNA endonuclease assay) with mutations confirmed by direct sequencing [101]. In a comparison of direct sequencing, FASAY and DNA microarray analysis of clinical samples from lung biopsies, the FASAY was used to determine the 'true frequency of p53 mutations' and the analysis of a subset of these was then performed by direct sequencing and a PCR/ligase detection reaction analysed by a DNA microarray. The results demonstrated that the microarray-based approach could provide high throughput and also was more sensitive than direct sequencing [102]. Other studies have evaluated similar DNA microarrays, such as resequencing arrays, and have found that these may be useful for large-scale mutational identification in clinical samples [103,104]. Ultimately, whichever assay may be used to analyse clinical samples, direct

sequencing of PCR products either from the tissue, or from assay products as in the case of the FASAY, is required to confirm the identity of the mutations.

The not very surprising take home message from such studies of tissues is that mutations of p53 occur in erstwhile normal tissues and using ultra-sensitive techniques such as single cell PCR can give rise to false positives with respect to detecting potential clones of cancerous cells. This can be contrasted with studies using the FASAY and direct sequencing, which have been used successfully to identify p53 mutations in cancers without detecting presumed false positives [94,105,106].

Whereas the detection of a single mutant cell may have little biological significance, the consequences of detecting a p53 mutation in a colony of cells would be expected to be of greater importance, possibly indicating the presence of a developing tumour. Colonies of cells have been identified in skin, for example (by virtue of expressing high levels of p53 protein), which in > 50% of cases harboured mutations in p53 [107]. The presence of such lesions in morphologically normal cells was not indicative of future cancer. To date, epidermal clones of p53 mutant cells have not been shown to harbour the same mutations as nearby cancers. Thus, there is no proof that these clones represent developing tumours, although it should be noted that these clones of cells do arise most often in skin that has been exposed to UV irradiation over long periods of time. That detection of p53 mutation in colonies of cells is not predictive of cancer may partly be due to the nature of the mutation detected. A considerable body of data has been generated that demonstrates the importance of knowing the identity of the p53 mutation for prognostication [108,109]. Recent studies in several cancers have convincingly demonstrated that missense mutations in the core domain of p53 involved in binding to DNA (either through contact with the DNA or through influencing the structure of the DNA binding region) are associated with more aggressive disease than those in less functionally critical regions of p53 [101,108]. Perhaps surprisingly, given other recent work that has demonstrated gain of function of a subset of these mutations [110,111], nonsense mutations and deletions of the p53 locus on 17p were found to be associated with disease that progresses at least as, if not more, aggressively [101,108].

One issue to consider in attempting to reconcile the clinical and experimental data regarding gain of function mutations is that only a very small number have been studied and thus it remains unclear to what extent these contribute to the cancer phenotype. Moreover, there are additional data that support the existence of a critical role for TP53 nonsense mutations in promoting disease progression. Analysis of the IARC TP53 database has shown that nonsense mutations of p53 occur approximately twice as often in cancer as might otherwise be expected and thus loss of function confers a selective advantage to the tumour cells [109]. It is important to remember that TP53 is unusual among tumour suppressor genes in that it has such a high frequency of missense mutations. The same study concluded that the most common events driving tumorigenesis with respect to p53 were loss of transcriptional activation, and to a lesser extent, acquisition of dominant negative activity [109].

Notwithstanding the above, the high frequency of hot spot mutations (nine missense mutations account for roughly 30% of all cancers) clearly demonstrates that not all mutations are equal. For these reasons, the use of p53 mutational detection as a future

diagnostic tool will need to be informed by an increasing body of information on the consequences of the specific mutation detected. For specific information on mutant effects, see the IARC TP53 database [112]. Status has been shown to impact on disease progression and thus it is logical to expect that diagnosing the status of the TP53 gene will become increasingly important in determining the management of patients with cancer. A good example of a disease where this is already happening is chronic lymphocytic leukaemia (CLL). For asymptomatic patients with low tumour burden at the time of diagnosis, the normal management remains 'watchful waiting' [113]. In some centres a panel of molecular markers are analysed that can predict the kinetics of disease progression, such as IgV_H mutation, CD38 and ZAP-70 expression [114]. However, for patients who have progressed to needing treatment, it is becoming increasingly routine to perform fluorescence *in situ* hybridisation (FISH) for chromosome 17p. Loss of the TP53 gene on chromosome 17p is currently the single best predictor of failure to respond to standard chemotherapy with purine analogs or alkylating agents [115]. Recent studies suggest that such patients may benefit from alternative treatments, such as those based on alemtuzumab [116].

Another potential use for p53 investigation is in the detection of residual disease. Screening for p53 protein expression and mutations has been applied to tissue samples at surgical resection margins in head and neck cancer and also to the analysis of isolated tumour cells in the bone marrow of patients with breast cancer [117,118]. One of the problems reported for isolated cancer cells is that these can arise very early in tumourigenesis and it has been shown in lung cancers that these may not harbour p53 mutations, even though these were present in the primary tumour [119]. Nevertheless, p53 mutation detection in deep resection margins has recently been shown to be linked with increased risk of recurrence in surgically treated patients with squamous cell carcinoma of the head and neck, and this has proven to be an important observation [120].

3. Expert opinion

It may appear that the pace of progress in the use of p53 as a diagnostic tool has been disappointing [10,11], but examples such as CLL and work on the consequences of the nature of p53 mutations on outcome shows that, as expected [10], investigating such a critical tumour suppressor gene can provide important information both at the time of diagnosis and afterwards. The challenge is to match the appropriate tests to the clinical question and to incorporate the enormous amount of information on p53 mutations [109]. In some cases, such as CLL, this has already been accomplished to the point where a useful diagnostic test has been developed. For solid tumours, attempts have been made to detect p53 mutations in even some of the most hostile environments for samples such as the use of a modified FASAY for pancreatic juices [121]. These and many other examples provide evidence that we should expect more such developments. Regarding the methods used, one thing that becomes clear is the need to balance sensitivity and specificity. There are many highly sensitive methods but these are capable of detecting p53 lesions that will not develop into cancers, whereas less sensitive methods that are highly specific, such as the FASAY, have already demonstrated their utility in a range of cancer types. In addition, the importance of the nature/identity of the mutation in p53 is becoming increasingly apparent [101,108,109]. One interesting point here is that it is not presently clear how to reconcile the

consequences of nonsense mutations with those of gain of function missense mutations, both of which appear to confer a poor prognosis (gain of function of p53 has so far only been unequivocally demonstrated in transgenic mouse models and thus only a very limited number of mutants are currently proven to display gain of function). One major difference between studies of mutations in patients with cancer and studies of gain of function mutations in transgenic mice is that the latter express the mutant transgene from an early timepoint in development and then throughout their adult life, whereas the former typically acquire p53 mutations later on, although why this would lead to a mutation-specific difference is unclear. Nevertheless, it is apparent that there are mutations that have much less biological significance and this information is likely to be increasingly vital for prognostication.

The literature is awash with studies using immunohistochemical detection of p53, when it is clear that used in isolation, this is not a reliable tool. There is little justification in the twenty-first century for not at least combining p53 detection with other markers that add significantly to information on p53 functionality in a clinical sample, such as MDM2 and p21(CDKN1A) [122]. Of course as technology advances the simultaneous monitoring of multiple genes is being increasingly used. DNA microarray-based strategies, including resequencing, seem certain to play an increasing role in the future and have been used with some success already, but at present these approaches are not sufficiently cost-effective for routine use. Relatively low-tech, cost-effective assays exist for p53 investigation, such as FISH for 17p, direct sequencing and the FASAY, albeit that it is not easy to translate the FASAY into a routine clinical diagnostic tool. This is balanced by the power of this technique as a first round screen for complex clinical samples that contain a mixture of normal and cancer cells. It seems clear that as we continue to learn more about p53, and in particular, about what the likely consequences of different mutations in this gene are, we will find an ever greater need for information on the status of p53, not only for prognostication in advancing disease but also increasingly at the time of diagnosis.

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Figure 1. p53 is induced by a range of stresses and coordinates a wide range of cellular responses p53 can be stabilised and subsequently activated following phosphorylation through direct modification by a range of kinases including ATM, ATR, DNA-dependent protein kinase and casein kinase II. In addition, p53 can be activated following oncogenic mutations by induction of p14^{ARF} which protects it from ubiquitylation by MDM2.

Figure 2. The p53–MDM2 auto-regulatory feedback loop

High levels of p53 and MDM2 can occur transiently following genotoxic stress, but stable high expression of both proteins is prevented by the feedback of MDM2 upon p53. MDM2 promotes degradation of p53 and this then reduces transcriptional activation of the MDM2 gene by p53, bringing MDM2 levels down. How stable levels of both proteins are maintained in some cancers is unclear but factors such as HSP90 may bind to both proteins preventing degradation of p53 by MDM2. Other possibilities include MDMX expression, which can inhibit p53 but does not directly promote its degradation (although it can interact with MDM2 to promote this). This would not explain the high levels of MDM2 that can arise and does not explain the strong association between p53 and MDM2 dual upregulation observed in some cancers such as renal cell carcinoma.

Figure 3. The p53 FASAY can be used to detect mutant p53 from a mixture of normal and cancer cells that contains as little as 1 – 5% of mutant p53-expressing cells

Red (p53 mutant) colonies on the left are detected in a sample containing 10% p53 mutant and 90% p53 wild-type cells. Typically 5 – 10% mutant cells can be detected in this way but as little as 1% can be detected through sequencing of multiple colonies to identify rarer mutants. The panel on the right shows the result of p53 FASAY using wild-type p53. Note that low levels of red colonies appear on these plates and are due to either recombination of the plasmid used for the FASAY and to spontaneous mutations introduced during PCR amplification. These are readily identified following sequencing of the p53 cDNA in the red colonies.