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## The relevance of TP53 for CLL diagnostics

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Short Title: *p53 in chronic lymphocytic leukaemia*

Abstract: TP53 disruption in Chronic Lymphocytic Leukaemia has long been known to hold vital prognostic information as well as dictate the appropriate course of treatment to be followed for patients. TP53 status is commonly assessed by FISH for del(17p) and Sanger sequencing for *TP53* mutational analysis. At present, current screening methods for these mutations could miss diagnostically relevant mutations potentially leading to inappropriate treatment decisions. In addition low levels of mutations that are proving to be clinically relevant may not be detected with current techniques. This review looks at the structure, function and regulation of the TP53 protein, the mutations found in cancer and CLL, the relevance of TP53 disruption in CLL and the current screening methods for TP53 mutations including Next-generation sequencing.

Introduction.

### *Tumour Suppressor p53 (TP53) Gene.*

Tumour Suppressor p53 (p53) was first identified in 1979 when two independent groups published their findings on the interaction of a cellular protein of approximately 53-55 kDa and a large T-antigen of the Simian virus (SV40).<sup>1,2</sup> p53 was initially thought to act as an oncogene and it was only in the late 1980's that it was correctly assigned as a tumour suppressor gene and then coined as the "Guardian of the Genome".<sup>3</sup>

The protein p53 contains 393 amino acids and is encoded by the *TP53* gene in humans. It is located on the short arm (p) of chromosome 17 (17p13.1) and possesses 11 exons, of which 10 are coding (figure 1).

Structure and Function.

P53 is a sequence-specific DNA binding protein that regulates transcription. The protein consists of two N-terminal transactivation domains with a conserved proline-rich domain, a central DNA binding domain followed by a C-terminus. This encodes its nuclear localisation signals and a domain essential for transcriptional activity.

The p53 protein level is low in normal cells as it is maintained by a series of regulators such as *MDM2* (E3 ubiquitin protein ligase) which facilitates ubiquitin-mediated degradation.<sup>4</sup> The role of p53 is to control cell proliferation through its upregulation in response to cellular stress such as DNA damage and activation of oncogenes. When activated, p53 protein levels increase and cell phenotypes change to include apoptosis and elevated DNA repair.<sup>5</sup>

P53 binds strongly to several specific DNA sequences via two transactivation domains in its structure.<sup>6,7</sup> *MDM2*, a negative regulator of p53 binds to the transactivation domain and induces an alpha-helical conformation and results in blocking transcription.<sup>8</sup> By translocating these genes during periods of cell stress, they become active, producing proteins involved in cell cycle progression and apoptosis<sup>9</sup>. During genotoxic stress, p53 protein is phosphorylated by ATM (Ataxia Telangiectasia Mutated) kinase and prevented from undergoing *MDM2* -mediated degradation (figure 2). Under oncogenic stress, p53 is

protected from degradation by p14ARF (alternate reading frame tumour suppressor) protein.

P53 is known to have a number of functions but the ability to promote cell cycle arrest and apoptosis are the best understood. It has been well recognised that p53 is capable of upregulating the cyclin-dependent kinase inhibitor CDKN1A. It is also well established that the transcriptional activity of p53 is tissue specific and prominent in lymphocytes.<sup>10</sup> P53 can also trigger apoptosis by relying on the induction of pro-apoptotic *BCL-2* family members facilitating caspase activation and cell death.<sup>11</sup>

Origins of p53.

*TP53* is a member of a broader gene family including *TP63* and *TP73*. Unlike *TP53*, both *TP63* and *TP73* do not have tumour suppressive capabilities. In eukaryotes the p53 protein sequence is relatively conserved, however p53 has evolved by increasing gene dosage.

At first, this critical anti-cancer gene was believed to be an oncogene due to its overexpression in many cancers such as breast and lung.<sup>12 13 14</sup> It was subsequently realised that this gene is mutated in half of all human cancers and the loss of regulatory function causes leads to oncogenesis.<sup>15</sup> p53 mutations also occur in the germline and are pathogenic in cases of Li-Fraumeni syndrome, a hereditary cancer predisposition syndrome.<sup>16</sup> The majority of tumour suppressor genes are frequently inactivated by deletions or truncating mutations causing a loss of protein expression. In contrast, the *TP53* gene is unique in comparison to other tumour suppressors as inactivation on both alleles is not required to eliminate p53 function.. Loss of function of *TP53* is considered a major event in tumourigenesis as well as being linked to resistance to chemotherapy and poor prognosis in many cancers.<sup>15</sup>

The most common and well characterised mutations are missense mutations within the DNA binding domain which occur in approximately 75% of tumours with approximately 25% of these falling into 6 hotspot codons. Interestingly 25% of *TP53* mutations are nonsense or frameshift mutations, whilst the remainder are splice site single nucleotide variants and in-frame indels. Inactivation of the second *TP53* allele occurs most commonly through loss of heterozygosity.<sup>17</sup>

A significant body of evidence suggests that certain mutant *TP53* alleles have a gain of function (GOF) property. This was first suggested over 25 years ago with the introduction of mutant p53 cells giving rise to a new phenotype distinct from null cells.<sup>18</sup>

As previously stated the *TP53* gene is mutated in half of all human cancers, however different tumours show a wide spectra of *TP53* mutations. The effect of mutant p53 has largely been assigned to two separate categories:

1. Those affecting amino acids that contact DNA preventing wild-type transcriptional activity with major disruption to the protein and
2. Those disrupting the three-dimensional structure of the protein.

Although mutations in this gene are most commonly seen in solid tumours, haematological cancers have also been shown to possess these mutations at a lower rate. However, one

common blood cancer, Chronic Lymphocytic Leukaemia (CLL), contains an unusually high number of TP53 aberrations as well as evidence for their role in disease pathogenesis.<sup>19 20</sup>

### *1.2. Chronic Lymphocytic Leukaemia (CLL).*

CLL is the most common leukaemia in adults in the Western world and is characterised by clonal expansion of CD5+ B cells, derived from healthy CD5+ B cells throughout the blood, lymphoid tissues and bone marrow of patients.<sup>21</sup> CLL has a highly variable presentation and clinical course. The majority of patients are asymptomatic, have indolent disease and are managed on an active surveillance approach without requiring treatment. A proportion of patients require therapy immediately after diagnosis and can have a rapidly progressive disease leading to early death. This clinical heterogeneity highlights the need for both prognostic and predictive markers in CLL.<sup>22</sup>

Considerable advances have been made over the past decade using genome-wide sequencing approaches to decipher the genetic complexity of CLL. However, the key therapy decision-making biomarkers in CLL are TP53 aberrations: chromosomal defects of 17p13 and *TP53* gene mutations.<sup>23 24</sup>

#### *TP53 aberrations in CLL Patients.*

*TP53* gene defects in CLL were first described in the early 1990's and the association with inferior clinical survival was established.<sup>19</sup> Initially, the loss of the *TP53* locus was not considered to be an important event in the era of karyotyping.<sup>25</sup> However, this was to change with the publication of Dohner's hierarchical CLL classification in 2000.<sup>26</sup> This firmly established the inferior survival of patients with deletions of 17p13 [del(17p)]. This was subsequently confirmed in other studies and implemented into routine practice.<sup>27</sup> Whilst karyotyping required dividing cells, Dohner's classification used Fluorescence *in situ* hybridisation (FISH). FISH uses fluorophore-labelled DNA probes that hybridise to specific DNA sequences that allows the identification of deletions, additions and translocations. In contrast, *TP53* mutational status was not initially assessed routinely in CLL diagnostics as it was assumed that *TP53* mutations did not occur in the absence of del(17p).<sup>28 29 30</sup> With further studies it became clear that del(17p) is usually accompanied by *TP53* mutations in CLL and studies have shown that both can occur independently with the frequency dictated by the particular patient cohort studied and the methodology used.

The frequency of *TP53* mutations are low in treatment naive patients (approx 10%) but this increases (up to 25-50% depending on study) in patients with disease progression and treatment refractory patients suggesting these alterations are mostly acquired and provide a mechanism of resistance.<sup>30</sup>

In general 90% of patients with del(17p) carry a mutation and up to 65% of patients with a *TP53* mutation have del(17p).<sup>31 32 33</sup> It has also been shown that monoallelic *TP53* mutations have an inferior progression and overall survival.<sup>31 33</sup> Of all patients with *TP53* disruption, mutations and deletions occur together in the majority of patients with monoallelic *TP53*

mutations accounting for another 40% of patients, monoallelic deletions and also mutations with copy neutral loss of heterozygosity (cnLOH) account for a minority of patients (figure 3).

TP53 as a treatment response predictor.

For more than a decade chemoimmunotherapy (CIT) in the form of fludarabine, cyclophosphamide and rituximab (FCR) was the conventional first line regimen in fit patients providing a high response rate, improved progression free survival and an impact on overall survival for the first time in CLL. Not all patients benefit from this treatment, with up to 25% of patients relapsing within 24 months, fulfilling the definition for chemorefractoriness.<sup>34</sup> Fludarabine, a purine nucleoside analogue, interferes with DNA synthesis and repair in dividing and non-dividing cells and therefore requires a functional p53 protein to exert its effects. The presence of TP53 aberrations reduces progression free survival as well as inferring chemotherapy resistance.<sup>29 30</sup> The advent of novel treatment options in CLL targeting the B cell receptor and anti-apoptotic molecules targeting BCL-2 have proved beneficial in the treatment of patients with TP53 aberrations providing alternative methods for cell death.<sup>35 36 37</sup> Therefore, this highlights the need for accurate assessment of TP53 defects to identify patients that will benefit from novel therapies.

Current Testing Regimens in Routine practice.

Current guidelines recommend that TP53 aberrations should be assessed in patients requiring therapy and due to clonal evolution they should be reassessed at each pre-treatment time point.<sup>24</sup> As already stated the assessment of del(17p) by FISH is routinely performed. The cut-off for a positive result varies within laboratories with the widely accepted threshold that >20% of cells should contain del(17p) to be a clinically relevant clone.<sup>38</sup> Sanger sequencing is widely used for *TP53* mutational analysis however it may misclassify cases of *TP53* mutations as wildtype when variants with allelic burdens below the detection limit of sanger sequencing are present. With the implementation of next generation sequencing (NGS) large amounts of DNA can be sequenced in parallel at a lower cost than the gold standard Sanger sequencing. Updated recommendations from the TP53 network of ERIC suggest the recommend threshold for reporting mutations detected by NGS should be approximately 10% of allelic burden.<sup>39</sup>

Targeted NGS panels are also available whereby custom probes are designed to target genes/regions of interest within the genome with much greater sensitivity. This targeted approach facilitates a greater depth of sequencing as a much smaller percentage of the genome is interrogated.<sup>40 41</sup> This technique has led to improved knowledge on gene mutations and signalling pathways by screening large cohorts.<sup>40 41</sup> For targeted NGS, if solely one time-point is available, intraclonal dynamics can be studied using the variant allele frequency (VAF) to infer the timing of mutations.<sup>43</sup>

The general principles of targeted NGS panels are based on sequencing of an amplified DNA template. As with other applications the DNA template is a double-stranded DNA construct consisting of short fragments of DNA flanked by adapters of known sequence. Illumina

sequencing is the most broadly used NGS technique and consists of a solid phase amplification of the DNA template on a flow cell with subsequent sequencing-by-synthesis.<sup>44</sup> Novel approaches such as capture based sequencing allows the simultaneous detection of somatic mutations, copy number variations and chromosomal translocations.<sup>45</sup> As with all technologies several limitations exist. One of the major limitations of NGS is the lack of standardised bioinformatics pipelines and the requirements to handle large data sets.<sup>46</sup>

As *TP53* mutational analysis has a major impact on treatment decisions, international collaborations such as The European Research Initiative on CLL (ERIC- [www.ericcll.org](http://www.ericcll.org)) have developed a network of TP53 reference centres within various countries. ERIC aims to provide standardisation and harmonisation of diagnostic strategies for TP53 mutational assessment resulting in robust testing strategies and offering quality assurance schemes.

The introduction of NGS allows the detection of *TP53* mutated subclones. Subclonal is used to describe variants that are not present in the entire tumour population. This has great clinical relevance in CLL as the disease progresses, different leukaemic cells acquire mutations leading to numerous subclones developing. The subclones are intrinsically linked to their environment, and any changes to this environment such as chemotherapy, create changes in the cells, allowing outgrowth of resistant clones.<sup>20</sup> This is particularly relevant in CLL as *TP53* mutated subclones occur in a significant proportion of cases and have the same unfavourable prognostic impact as that of clonal *TP53* defects.<sup>47 48</sup>

In CLL the inactivation of the *TP53* gene by either a deletion or a mutation has a negative impact on the disease course for patients. TP53 aberrations are markers of chemorefractoriness and treatment decisions are based on this finding. TP53 aberrations should be tested before each treatment decision and NGS represents an ideal technique for mutation assessment due to detection of clinically relevant mutated subclones.

## Statements:

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## Figure legends:

Figure 1. Schematic structure of *TP53* gene. The *TP53* gene is located on the short arm (p) of chromosome 17 (17p13.1) and possesses 11 exons, of which 10 are coding.

Figure 2. p53 function and regulation.

*MDM2*, mouse double minute 2; UV, ultraviolet, *ATM*, ataxia telangiectasia mutated

Figure 3. *TP53* defects: Loss of p53 function in CLL can occur due to del(17p) and/or *TP53* variants. The most common cause of *TP53* aberrations results in the combination of del(17p) and *TP53* mutation.

