Chapter 5
TP53 Aberrations in Chronic Lymphocytic Leukemia

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Abstract  CLL patients harboring TP53 defects remain the most challenging group in terms of designing rational and effective therapy. Irrespective of the treatment employed—chemotherapy, chemoimmunotherapy, or pure biological drugs—median survival of these patients does not exceed 3–4 years. This adverse outcome is caused by a less effective response to therapeutics acting through DNA damage induction and relying on the subsequent initiation of apoptosis as well as by virtually inevitable aggressive relapse. Patient proportions with TP53 defects at diagnosis or before first therapy were reported within the range 5–15 %, but they increase dramatically in pretreated cohorts (reported up to 44 %), and also in patients with Richter transformation (50 % harbor TP53 defects). Currently, most laboratories monitor TP53 defect as presence of 17p deletion using I-FISH, but 23–45 % of TP53-affected patients were shown to harbor only mutation(s). In other patients with intact TP53, the p53 pathway may be impaired by mutations in ATM gene coding for the p53-regulatory kinase; however, prognosis of ATM-defective patients is not as poor as those with TP53 abnormalities. Though many novel agents are under development, the monoclonal antibody alemtuzumab and allogeneic stem cell transplantation remain the basic treatment options for TP53-affected CLL patients.

Keywords  TP53/p53 mutation • Deletion 17p • Apoptosis • Prognosis • Relapse • Chemo-refractoriness • Alemtuzumab • ATM
Tumor Suppressor p53

p53: A Tumor Suppressor with Unique Properties

In 1979, two independent research groups [1, 2] published their reports noting a physical interaction between a large T-antigen of the SV40 virus and a cellular protein of approximately 53–54 kDa. This is when the fascinating story of p53 research commenced. Although this yet unknown protein attracted a lot of attention from the very beginning, determination of its principle role in tumor cells has not always been straightforward. Still, in 1984, p53 had been erroneously assigned among oncoproteins (for review see [3]). This incorrect classification was influenced by two basic factors: (a) protein accumulation was frequently observed in tumor cells, by contrast to normal cells, which resembled an oncogenic behavior, and (b) complementary DNA (cDNA) clones used for transfection experiments into human cells harbored missense mutation, and—as disclosed later—some missense mutations exert the ability to switch the p53 from a tumor suppressor to a powerful oncoprotein. Only later studies from the end of 1980s confirmed the tumor-suppressive behavior of wild-type p53 [4, 5] and, finally, 13 years from its discovery, the p53 protein was officially proclaimed as the “Guardian of the Genome” [6].

Although p53 activity was recently shown to impact the pathogenesis of several nonmalignant diseases, the p53 role in cancer prevention is substantially more elaborated. Currently, both experimental data and clinical observations recognize the p53 as the most important tumor-suppressor protein: (a) $TP53^{-/-}$ mice invariably develop tumors [7], (b) heterozygous inherited mutations predispose to the Li-Fraumeni cancer-prone syndrome in humans [8], and (c) somatic mutations are frequent in many different types of human tumors [9].

The p53 plays a critical role in an anti-cancer barrier preventing an organism from malignant cell proliferation [10–12]. During early cancerogenesis, tumor cells experience genotoxic stress, which elicits a DNA damage response (DDR) pathway—the hierarchically ordered machinery detecting DNA lesions and signaling their presence to protein complexes that either promptly repair the damaged DNA or arrest the cell cycle if DNA repair requires additional time. Alternatively—in case when DNA damage is too extensive and repair is not possible—the DDR pathway induces apoptosis or replicative senescence. For effective induction of the above-mentioned processes, p53 activity is crucial.

Considering these facts, it is not surprising that the central axis of the DDR pathway, involving both the p53 and its positive regulator, the ATM kinase (Ataxia Telangiectasia Mutated), is under enormous pressure to be impaired during malignant conversion [11, 12]. Indeed, $TP53$ mutations in particular are frequent in many different tumors and most often observed in ovarian, colorectal, and esophageal cancer [13]. Even a low $TP53$ mutation frequency, which is typical for some types of tumors, may not mean that p53 is irrelevant in prevention of their development. In cervical carcinoma, for instance, a typically non-mutated p53
protein is inactivated by direct physical interaction with an E6 oncoprotein encoded by the high-risk human papillomavirus (type 16 or 18) [14]. In other tumors with an intact TP53 gene, the p53 pathway may also be abolished by enhanced activity of p53 inhibitors (e.g., MDM2) or defects in upstream p53 activators (such as ATM in CLL) or downstream target genes (e.g., inactivation of NOXA and others in different B-cell lymphomas [15]). Frequency of p53 inactivation in hematological malignancies is lower in comparison with solid tumors, usually reaching 10–15% in unselected patient cohorts. However, in contrast with the solid tumor situation, virtually all relevant studies agree with a severe prognosis for patients with hematological malignancies and p53 inactivation [16].

**p53 Protein Structure**

The TP53 gene is located at the short arm of chromosome 17 (17p13.1) and contains 11 exons, 10 of which (2–11) are coding. The full-length protein consists of 393 amino acids and harbors several structural domains: (a) the N-terminal domain, which ensures target gene transactivation; (b) the central DNA-binding domain, which directly interacts with consensus DNA sequence in the target promoters; (c) the oligomerization domain, through which the four monomeric polypeptide chains join together to form a final tetramer molecule; and (d) the C-terminal domain, which harbors important regulatory sites for the DNA-specific and also nonspecific p53 binding. Besides the basic full-length protein, analysis of TP53 gene sequence also revealed 12 putative p53 isoforms (for review see [17]). Expression of individual isoforms is tissue-specific, and they differ in subcellular localization. In quiescent lymphocytes, isoform p53β is typically expressed [18].

The TP53 gene contains many polymorphisms, with 11 of them being non-synonymous. The most frequent polymorphism P72R (changing the ancestral proline allele to arginine, which is notably more frequent in some populations) was described to have functional impact, and its role in cancer susceptibility, prognosis, and treatment response was studied in several malignancies with inconsistent results (for review see [19]). Similarly, in early CLL studies no relation between this polymorphism and clinical outcome was evident [20, 21]; in a more recent study, the proline allele in the homozygous state was associated with a shorter time to first treatment among the group of patients with mutated IGHV locus [22].

**Regulation of p53 Protein**

The p53 protein level in a cell is low under normal conditions—undetectable by western blot or immunohistochemistry. This is because the p53 induces—among other targets—expression of MDM2 gene coding for a protein which serves as a negative regulator, targeting p53 protein for ubiquitin-mediated degradation
p53 protein stabilization following genotoxic stress is then ensured through delicate posttranslational modifications namely involving phosphorylations, but also acetylations or sumoylations [23]. The p53 Ser-15 phosphorylation elicited by ATM kinase is critical for p53 protein stabilization after induction of DNA double-strand breaks (DSBs) [24, 25]; this phosphorylation prevents MDM2 binding to p53. Another situation arises in case of oncogenic stress (activation of oncogenes, e.g., BCR-ABL1), when p53 is stabilized by the product of a CDKN2A gene, i.e., p14ARF protein that directly inhibits p53-MDM2 binding [26] (Fig. 5.1a).

**Fig. 5.1** Overview of p53 stabilization (a) and activity (b). In case of a genotoxic stress the p53 protein is phosphorylated by ATM kinase and thus prevented from MDM2-mediated degradation. Under oncogenic stress p53 is protected from degradation by p14ARF protein. After stabilization the p53 protein regulates through transcriptional activation of target genes several divergent but interconnected processes, which are decisive for a cell fate. The p53 also localizes on mitochondria, where it interacts with Bcl2 protein family members. This translocation contributes to permeabilization of the mitochondrial membrane, cytochrome c release, and subsequent apoptosis (Fig. 5.1a).
Cellular Functions of p53

The p53 is a transcription factor with a consensus binding site consisting of two copies of the 10 base pair motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0–13 base pairs [27]. It is estimated that p53 regulates more than 125 structural genes [28]. The expression of many p53-regulated proteins is critical for decisions made within the DDR (Fig. 5.1b). Regarding cell cycle arrest, the cyclin-dependent kinase inhibitor CDKN1A (coding for p21 protein) seems to be the most prominent target upregulated by p53, while PUMA (p53-upregulated modulator of apoptosis) is a key mediator of p53’s apoptotic activity [28]. Transcriptional activity of p53 is tissue-specific [29] and is distinct in lymphocytes compared to other cells [30]. Among p53 target genes there are also numerous microRNAs, including miR-34a, with a proposed role in CLL pathogenesis [31, 32]. Recently, p53’s role in posttranscriptional maturation of several microRNAs with growth-suppressive properties (including, e.g., miR-16) has been reported [33].

How p53 can discriminate between cell cycle arrest, senescence, or apoptosis still remains a matter of intense debate [34]. The p53 molecule conformation represents perhaps the most critical factor, and this conformation depends on two basic events: (a) posttranslational modifications, such as phosphorylations, methylations, or acetylations; and (b) DNA-binding itself that determines which cofactors will participate in target gene transactivation and thus influence transcription extent.

The p53 is also able to trigger apoptosis independently on transcription initiation. This mechanism facilitates a fast response to genotoxic stress, when p53 localizes on mitochondria, it interacts with Bcl2 protein family members, enabling oligomerization of bak and bax proteins and subsequent permeabilization of the mitochondrial membrane and cytochrome c release, which leads to caspase cleavage (Fig. 5.1b, for review see [35]). It was even suggested by Steele et al. [36] that transcriptional-independent apoptosis is a major route to cell death induction by p53 in CLL cells, as they showed that blocking of p53-mediated transcription paradoxically augmented apoptosis induction by chlorambucil and fludarabine through accelerating the proapoptotic conformation change of the Bax protein.

Another important p53 cytoplasmic function is inhibition of autophagy through mTOR pathway promotion. Interestingly, p53 plays an ambiguous role in autophagy control, as nuclear p53 can induce autophagy through transcriptional activation of positive autophagy regulators (including mTOR inhibitors) (for review see [37]). Autophagy is tightly connected with apoptosis, although their exact interplay remains a matter of debate. Autophagy inhibition facilitates cell death, yet on the other hand, its activation promotes the cell’s attempt to cope with stress and to survive. However, with excessive autophagy, an autophagocytic death via necrosis may occur. Therefore, the p53 decision between autophagy induction and inhibition is crucial for cancer treatment, and autophagy was identified as an important mechanism of drug resistance. Early studies also suggested its relevance in CLL resistance to treatment by flavopiridol [38] or dasatinib [39].
In contrast with certain other tumor-suppressors, the \( TP53 \) gene doesn’t need to be inactivated on both alleles to eliminate p53 function. The p53 pathway is highly sensitive to p53 protein level changes, and the protein produced by only one allele may not be sufficient to ensure proper function. This effect, termed “haploinsufficiency,” was documented on mouse models [40] and is assumed in patients with sole 17p deletion (del(17p)) or sole truncating mutation. However, simple loss of function (LOF) may not be the most grievous p53 defect contributing to tumor progression. This could have been predicted from the highly predominant occurrence of p53 missense substitutions (forming approximately 75 % of all mutations) leading to expression of aberrant protein in cancer patients. Indeed, besides LOF, another two effects clearly attributable to mutated p53 have been evidenced: (a) dominant-negative effect (DNE) of monoallelic mutation towards the second intact (wild-type) allele, and (b) gain-of-function (GOF) effect probably acting independently on the allele status.

DNE is most likely caused by final p53 molecule inhibition through hetero-oligomerization of mutated and non-mutated p53 polypeptide chains. The p53 functions as a tetramer composed of a dimer of dimers with co-translational forming of individual dimers. Once one p53 allele is mutated in the DNA-binding region, half of the dimers would be active, the other half would be inactive, and final post translational tetramerisation would render inactive 75 % of p53 tetramers [41]. Accompanying del(17p), frequently observed in mutated patients, then probably eliminates the rest of p53 activity. By contrast, a sole del(17p) eliminates only 50 % of p53 molecules, and this may potentially explain why monoallelic missense mutation, but not sole del(17p), is frequently selected in CLL patients [42].

In addition to the DNE, it has also been well evidenced through numerous studies and several experimental systems that some missense mutations exert a strong GOF effect. This effect was initially demonstrated in cell lines lacking endogenous p53, when the mutated \( TP53 \) gene had been expressed and the phenotype did not copy a simple loss of p53 function [43]. A pivotal mechanism of the mutated p53 GOF seems to be an interference with the p53-related proteins, i.e., p63 and p73, which prevents their tumor-suppressive functions [44]. In addition, some p53 mutants have been shown to upregulate genes and miRNAs, supporting cancer progression or precluding effective therapy. For instance, mutated p53 was shown to enhance the expression of multidrug-resistance gene 1 (MDR1) [45]. A number of other GOF mechanisms were described (for review see [46]) including direct protein–protein interactions, e.g., interaction with NF-\( \kappa \)B leading to prominent enhancement of a cancer progression [47], or interaction with the nuclease Mre11, which suppresses the binding of the Mre11-Rad50-NBS1 (MRN) complex to DNA DSBs, leading to impaired ATM activation [48]. Mutated p53 has also been implicated in abrogation of the mitotic spindle checkpoint [49].
GOF are mutant and cell-type-specific, and preliminary data suggests that a strong mutated p53 GOF effect may also be present in CLL patients with particular p53 mutations [50].

**p53 Activity: More than Cancer Protection**

Despite its indisputable role in protecting an organism from developing a tumor, p53 protein activity may not always be desirable. One example might be the adverse effect of anti-cancer therapy (namely chemotherapy and radiotherapy) on normal tissues; in this respect, a transient p53 inhibition during therapy could be a reasonable way how to protect healthy cells from unwanted apoptosis [51]. Recently, p53 protein contribution to the pathological elimination of nonmalignant cells is gradually being recognized. Specifically, p53 protein activation has been proven to result in cardiomyocyte necrosis during ischemic heart disease, and p53-dependent apoptosis then appears to lead to the pathological neurodegeneration in Alzheimer’s, Parkinson’s, and Huntington’s diseases [28].

**p53 Mutation Functional Impact Assessment**

While some *TP53* mutations manifest obvious effects on p53 function (e.g., nonsense or frame-shift mutations which abolish the DNA-binding domain), identified missense substitutions should always be checked for severity and predicted clinical impact. Comprehensive analysis of individual *TP53* missense mutations is available via the web pages of the International Agency for Research on Cancer (IARC; http://www-p53.iarc.fr). The following statistics are available in this database: (a) mutated protein activity assessed towards eight selected target promoters (percentage in comparison with wild-type protein); this activity was analyzed using a yeast functional assay and is available for all potential 2 314 p53 missense mutations [52]; for some mutants, information about functionality in human cells is also available; (b) structural impact of mutations; (c) available data about DNE and GOF; (d) frequency of particular mutations in cancer patients and Li-Fraumeni families; (e) list of described p53 polymorphisms.

**TP53 Gene Abnormalities in CLL**

The frequency of *TP53* defects in hematological malignancies varies between 5 and 20 %, which is low in comparison with solid tumors, where it can reach up to 80 % (http://www-p53.iarc.fr); however, their impact on disease course is unequivocal, with p53 abnormalities having a well-documented role in chronic lymphocytic
leukemia. Within 1–2 years, patients with TP53 defects almost uniformly require treatment, are often chemo-refractory, and their expected survival is distinctively reduced, with almost exclusive disease-related death. CLL patients with TP53 gene defects were therefore assigned to a small but challenging subgroup of patients that was defined as “ultra high-risk CLL” [53].

History of TP53 Gene Defects Examination

Initial TP53 gene defects in relation to CLL were reported as early as in 1991, when Gaidano et al. [54] identified TP53 mutations in different human lymphoid malignancies and described their presence in late CLL stages. Subsequent studies showed the presence of TP53 mutations in 10–15 % of patients, confirmed association with advanced stages [55], and delineated the association of mutations with chemo-refractoriness and poor clinical outcome [56]. Loss of the TP53 locus (del(17p)) was not considered an important recurring event in CLL during early studies using conventional karyotyping, which most distinctively identified trisomy of chromosome 12 [57, 58]. Significance of del(17p) was proven only when interphase fluorescence in situ hybridization (I-FISH) was introduced [59]. Adverse prognostic impact of del(17p) was definitely confirmed in the year 2000 by Dohner et al. [60]. In this study, a comprehensive set of FISH probes was employed with del (17p) being the strongest predictor of poor survival and reduced time to treatment, followed by 11q deletion and trisomy 12; deletion 13q as the sole abnormality exhibited the best prognosis. Based on this observation, a hierarchical prognostic stratification model was suggested that is still referenced today, and assessment of del(17p) using I-FISH was introduced into general practice. Recommendation for a del(17p) examination, at least in clinical trials, was also included in a report from the International Workshop on Chronic Lymphocytic Leukemia (IWCLL) updating guidelines for the diagnosis and treatment of CLL [61].

The clinically relevant cut-off value for del(17p) presence has long been sought after. In the LRF CLL4 trial comparing Chlorambucil (Chl), Fludarabine (F) or Fludarabine, and Cyclophosphamide (FC), no difference in progression-free survival (PFS) or response duration was observed between the patients having 5–20 % of cells with del(17p) and those without this deletion. It was therefore suggested to use 20 % positivity as a clinically relevant cut-off for del(17p) [62]. However, there is still insufficient evidence to use the uniform clone size cut-off in diagnostic procedure.

While the assessment of the del(17p) presence is relatively easy, sensitive, and provides quantitative information on the proportion of affected cells, examination of TP53 gene mutations is more complicated, and no standardized approach for CLL patients has been established. Initially, it was assumed that correlation between deletion and mutation of the second allele is high in cancer cells [63], although some reports noted a common presence of sole mutation in later stages of tumor development [64]. Currently, most laboratories still investigate the TP53
defect in CLL patients only as del(17p) by I-FISH both in routine clinical practice and in clinical trials according to official worldwide recommendations [61]. **TP53** mutations only became a renewed subject of interest after several studies showed that quite a large proportion of patients carry TP53 mutations in the absence of del(17p) and that such mutations have an independent prognostic impact [42, 65–69]. Based on these observations, the European Research Initiative on CLL released the recommendations on TP53 mutation analysis in CLL in 2012 [70]. Thus, examination of both mutations and deletions is currently recommended before any treatment initiation.

**Prognostic Impact of 17p Deletions and TP53 Mutations**

Despite rare cases with del(17p) and indolent disease course (usually manifesting a mutated IGHV status) [71, 72], a strong adverse impact of del(17p) was observed in numerous studies and clinical trials. With these patients both a short PFS and overall survival (OS) were documented [73, 74]. The p53-affected patients exhibit markedly poor responses to various chemotherapy-based regimens involving alkylating agents or purine analogues [75–78] as well as their combination with the anti-CD20 monoclonal antibody rituximab [79, 80]. Even chemoimmunotherapy involving rituximab in combination with fludarabine and cyclophosphamide (FCR), which is currently considered the first treatment option for physically fit CLL patients, did not abrogate the negative del(17p) prognostic effect. Although patients with this genetic abnormality enrolled in a CLL8 study comparing FC and FCR manifested a prolonged PFS in the FCR arm (FCR: 11.3 months vs. FC: 6.5 months; HR 0.47), only 5 % of patients achieved complete remission after FCR therapy, and OS was significantly shorter in comparison with all other cytogenetic subgroups, reaching a median of approximately 3 years [81].

Deletion 17p is usually accompanied by TP53 mutation in CLL cells, but both sole del(17p) and sole TP53 mutations occur. Their frequencies and mutual proportion vary among different studies (Fig. 5.2). In addition, a mutation in the absence of del(17p) may be accompanied by uniparental disomy (UPD) resulting in duplication of the mutant allele [82]. The first study which examined the impact of mutations in the absence of del(17p) was performed on patients enrolled in US Intergroup E2997 trial (F vs. FC), and in contrast to del(17p) presence, no independent impact on PFS was observed for TP53 mutations. However, this output could be influenced by including polymorphisms and intronic as well as unconfirmed mutations in this study [76]. By contrast, later studies recorded a reduced time to first treatment [83], reduced PFS [68], and also adverse OS [42, 65, 68] in patients with sole TP53 mutations. Independent negative prognostic impact of TP53 mutations was also subsequently confirmed in prospective clinical trials [66, 69, 84].

A majority of mutations identified in CLL are missense substitutions localized in the DNA-binding domain with a mutation profile similar to other cancers, though several specific features were described (Fig. 5.3). A high incidence of an unusual
**Fig. 5.2** Frequency of TP53 defects and their composition in selected relevant CLL studies

**Fig. 5.3** Distribution of mutated codons in CLL patients based on the most comprehensive study by international collaborative group (n = 268 TP53 mutations) [86]. Structure of p53 protein with highlighted DNA-binding motifs inside the DNA-binding domain.
two-nucleotide deletion in codon 209 was documented [85] and later confirmed by an international collaborative study analyzing 268 mutations from four independent cohorts [86]. The same study also revealed a decreased percentage of transitions at CpG sites with bias favoring G-A exchange when compared to C-T exchange. Interestingly, G-A transitions were shown to be preferentially selected in quiescent, nondividing cells as a mirror reflection of cytosine deamination (C-T mutation) in the coding, non-transcribed DNA strand [87].

Data from in vitro studies suggest that not only the presence of a TP53 mutation, but also the type of mutation and its position matter. Indeed, it was shown in other malignancies that specific TP53 mutations are associated with either a poorer prognosis or a worse response to treatment than other TP53 mutations; however, the results are often contradictory due to the complexity of p53 pathway defects, unpredictable other genetic context, and lack of prospective studies [88–92]. Preliminary data in CLL [50] showed that patients with mutations in p53 DNA binding motifs (DBMs) (codons directly involved in DNA binding localized in loops L2 and L3 and in the loop-sheet-helix motif) have clearly reduced survival rates compared with patients carrying other p53 mutations. All mutations included in this study led to a basic loss of p53 transactivation activity. Substantially worse survival of patients with DBMs mutations may, therefore, most likely be attributed to a strong mutated-p53 GOF. It is important to study this potential phenomenon in CLL cells since there are innovative studies focusing on activation of p53 homologs in patients with a TP53 defect [93–95]. There might be a critical difference in utility of this approach between patients with absent p53 and those harboring p53 missense mutation connected to the GOF effect, which could potentially interfere with the activity of homologs.

**Clonal Evolution of TP53 Defects**

Frequency of mutations and deletions strongly varies depending on the disease stage and the cohort analyzed. At diagnosis, only 4.9 % of patients were reported to carry 17p deletion and/or TP53 mutation [96]. In untreated cohorts and in patients analyzed before first therapy the frequency varies between 8.5 and 14.8 % [66, 68, 69], and occurrence sharply increases after treatment, where it can reach up to 44 % in fludarabine refractory disease [31] and 50 % after CLL transformation to Richter syndrome [97] or to prolymphocytic leukemia [98]. The increasing proportion of TP53 abnormalities suggests that clonal selection of adverse genetic defects plays an important role during CLL progression. The Mayo Clinic report [99] illustrated the del(17p) selection during the CLL course and associated this selection with the high expression of zeta-associated protein (ZAP-70) and the presence of treatment. Another study [100] associated clonal evolution of del(17p) specifically with foregoing therapy presence. Several research groups analyzing p53 mutations then similarly documented their selection under therapy pressure [42, 65, 68, 101].
The higher frequency of TP53 mutations after therapy administration and also the higher frequency of transversions as opposed to transitions led to the notion that these mutations might directly be induced by chemotherapy [85, 102], similarly to what was evidenced with the chemical compound aflatoxin or tobacco smoke in other types of cancer [103, 104]. However, the most extensive collaborative study involving 268 p53 mutations didn’t record any differences in the mutation profiles of CLL patients with or without previous therapy [86], which indicated that treatment probably did not contribute to mutation origination. This view is currently supported by direct analyses of TP53 mutation presence in samples taken before therapy administration from patients who were later (after therapy) shown to acquire clonal TP53 mutation [65, 105].

**Association of TP53 Defects with Other Genetic Variables**

Abnormalities in the TP53 gene mostly occur in patients with unfavorable unmutated IGHV locus [73, 86] and constitute an independent adverse prognostic factor within this subgroup [50]. In line with the role of p53 as the guardian of the genome (see above), TP53 abnormalities were also associated with elevated genomic complexity in CLL patients [106, 107].

In 2001, Pettitt et al. [108] reported that CLL samples, which manifested p53 dysfunction (assessed through defective response to ionizing radiation) but did not harbor any TP53 defect, have ATM kinase impaired by a mutation. Since ATM is a positive regulator of p53 protein, it has been suggested that ATM inactivation may represent an alternative to p53 dysfunction. This notion has subsequently been confirmed in other study [109] and is usually observed in routine clinical practice when investigating del(17p) and del(11p) presence, since these two deletions are rarely observed in the same patient. Importantly, with respect to p53 dysfunction, these two defects should not be considered as equivalent, as the prognosis of ATM-defective patients is not as poor as those with TP53 abnormalities.

A polymorphism within the p21 gene has also been linked to p53 pathway dysfunction [110]. In addition, impaired p21 up-regulation despite an intact p53 response, which is not connected to any known gene defect, has been associated with early relapse [111]. Recently, mutations in the BIRC3 gene, a negative regulator of NF-κB signaling, have been shown as the cause of fludarabine chemo-refractoriness in CLL patients having an intact TP53 gene, with patient prognosis being as poor as with TP53-defective group [112].

**Current and Future Therapeutic Options for Patients with TP53 Defects**

Chemotherapeutic regimens based on alkylating agents and/or nucleoside analogues act through DNA damage induction and therefore require functional
p53 for efficient triggering of apoptosis. Consequently, pure chemotherapy and also its combination with rituximab proved to be ineffective in p53-affected CLL patients (see above; reviewed in [113]).

Currently these patients are treated most often with the anti-CD52 monoclonal antibody alemtuzumab, which functions independently of p53. Initially, effectiveness of alemtuzumab was noted in relapsed/refractory CLL, with the patients with del(17p) responding similarly to other patients [114–117]. A CAM307 study comparing alemtuzumab and chlorambucil as frontline therapy also yielded promising results in relation to treatment response, although response duration was very short in all patients, i.e., also in the alemtuzumab arm [118].

Subsequent trials combined alemtuzumab with chemotherapy and also non-chemotherapy agents. Alemtuzumab with fludarabine alone (FluCam) [119, 120] or in combination with other agents (cyclophosphamide—FCCam [121, 122], or cyclophosphamide and rituximab—CFAR [123]) were reported to produce high response rates in del(17p) patients, but such regimens show extended risk of toxicity. On the other hand, the combination of alemtuzumab with glucocorticoids appears to be meaningful, as glucocorticoids act independently of the p53 pathway, and high dose methylprednisolone (HDMP) on its own was shown to induce remissions in patients with TP53 abnormalities [124]. Glucocorticoids, by contrast to alemtuzumab, are effective in reducing lymphadenopathy and are able to wash out CLL cells from tissues into the bloodstream, where the cells can be more susceptible to alemtuzumab elimination. Accordingly, alemtuzumab in combination with methylprednisolone (CamPred) [125] or dexamethasone (CamDex) [126] was proven to be highly effective for del(17p) CLL patients. Glucocorticoids were combined not only with alemtuzumab but also with rituximab: R-HDMP [127, 128], R-dexamethasone [129], or humanized anti-CD20 antibody ofatumumab (O-dexamethasone); a phase II trial is ongoing and will be completed soon.

However, none of the currently approved therapeutic strategies noted above are able to attain long-term remissions in p53-defective patients. Therefore, ASCT still remains a viable option for young and physically fit p53-defective patients, with the potential to induce long-term disease-free survival [130, 131]. ASCT, however, is only available for a small subset of patients and is connected to nonrelapse mortality associated with development of graft-vs.-host disease (GVHD); both early and late relapses frequently occur.

A number of novel compounds are in various phases of clinical or preclinical testing. Biological targeted treatment functions via p53-independent triggering of apoptosis, which includes diverse mechanisms of action: (a) targeting of CLL cell surface molecules (CD20, CD23, CD37) by monoclonal antibodies or small modular immunopharmaceuticals (SMIP) [132]; (b) use of immunomodulatory and microenvironment modulating agents (IMiDs) [133]; (c) promoting the apoptotic pathway using, e.g., Bcl-2 antisense oligonucleotide, BH3 mimetics, or Bcl-2 inhibitors (for review see [134]); (d) altering histone modification by inhibition of histone deacetylases [135]; (e) targeting cell signaling through inhibition of cyclin-dependent kinases; [136] or by inhibition of the BCR signaling pathway—inhibition of NFκB and kinases PI3K-delta, SYK, AKT, Lyn, and mainly Bruton’s
tyrosine kinase—this approach belongs among the most promising, as it directly impacts B-cell proliferation and survival (for review, see [137]). Another approach is activation of p53 homologs in patients with a TP53 defect [93, 94] or direct targeting of mutant p53 protein and thus sensitizing the CLL cells to chemo/chemoimmunotherapy treatment using: (a) suppression of mutated p53 by antisense oligonucleotide [138]; (b) reactivation of p53 by specific compounds directed to wt or mutated p53 molecules [139]; (c) inhibition of heat-shock protein 90 that leads to destabilization of many tumor-promoting proteins, including mutant p53 molecules [140, 141].

Notwithstanding progressive scientific achievements, there is currently no optimal treatment available for CLL patients with 17p deletions and/or TP53 mutations. The patients still have dismal expectations and should be scheduled to participate in applicable trials whenever possible [61].

Techniques for TP53 Mutation Analysis

No standardized methodology is currently used for TP53 mutational analysis in CLL samples and individual centers utilize different approaches. A list of available methods with more detailed description is provided in ERIC recommendations on TP53 mutation analysis, published in 2012 [70].

Direct Sequencing

Direct (Sanger) sequencing is still one of the most often used methods. Genomic DNA (gDNA) rather than cDNA is preferred, as RNA-based analysis may omit some mutations which lead to nonsense-mediated mRNA decay (i.e., some nonsense or frame-shift mutations) [142]. The detection limit of Sanger sequencing may not be sufficient to detect small subclones with TP53 mutation, especially in cases without deletion of the second allele. Primers and reaction conditions can be found at the IARC p53 website (http://www-p53.iarc.fr/p53sequencing.html). Sequencing may be restricted to exons 4–9 or alternatively 4–10, as mutations in exons 2, 3, and 11 are very rare and exon 10 contains only about 4% of all mutations [86].

Prescreening Methods

The usage of prescreening methods such as denaturing high performance liquid chromatography (DHPLC) or high resolution melting (HRM) makes the mutational screening faster, cheaper, and more sensitive. However, identification of the particular mutation by Sanger sequencing is always essential. Primers and conditions for
DHPLC can be also found at the IARC p53 website. DHPLC can reach a sensitivity of up to 5–10 % of mutated alleles (depending on the particular sequence); however, mutation confirmation by the less-sensitive Sanger sequencing method is not always possible.

Another screening option represents the yeast functional assay (FASAY—Functional Analysis of Separated Alleles in Yeast) that directly identifies inactivating mutations, thus distinguishing them from silent alterations, polymorphisms, and partially or fully functional mutations. In this assay, the TP53 gene from patient cells is expressed in yeasts that function as reporter cells. FASAY is fast, cheap, and has a sufficient detection limit (10 %) [143, 144]. Underlying mutations should always be determined by sequencing. The mutation identification is based on DNA sequencing from yeast clones, which is more sensitive than direct sequencing of gDNA. However, since FASAY is a RNA-based method, it may not detect mutations leading to RNA degradation due to nonsense-mediated mRNA decay.

**Microarrays**

Microarray resequencing provides high sensitivity, with detection limits reaching up to 3 % of the mutated clone; however, this limit varies depending on the particular sequence. The Roche Amplichip p53 test, based on Affymetrix platform, is currently under development. The procedure is fast and user-friendly and is intended as in vitro diagnostic tool without need for confirmatory sequencing. This microarray was already tested in CLL studies [84, 145], but is not commercially available yet. The main shortcoming of microarray resequencing is the ability to detect only mutations for which the probes are printed on the array (for Amplichip, e.g., all single base pair substitutions and single nucleotide deletions in exons 2–11 and splicing sites).

**Functional Tests of the p53 Pathway**

Several partially modified tests have been suggested, based on DNA DSBs induction followed by the monitoring of p53 accumulation and subsequent p21 induction [108–111, 146, 147] or the induction of p21 together with other p53-downstream genes [146, 148]. An alternative approach utilized etoposide and nutlin-3a for efficient distinguishing of TP53 and ATM defects [149]. Another alternative represents the measurement of miR34a base level. In this assay, no cell treatment is required since the miR34a basal level is decreased in patients with p53 defects in comparison with patients carrying functional p53 [31, 32]. Although a functional assessment of the p53 pathway seems to be an elegant means of identifying potential TP53 and ATM defects, this testing is tricky and partially provides inconsistent results in relation to cell abnormalities [146] which still precludes its application in routine diagnostics.
Next-Generation Sequencing

Current rapid development of next-generation sequencing (NGS) technologies has allowed their utilization within a wider scientific community. Initial upfront cost of instrumentation is still very high, but with high throughput of samples or more screened target genes the methodology becomes cost-effective. Usage of highly sensitive technologies such as ultra-deep sequencing allows a detection of very small clones carrying TP53 mutation, but the clinical impact and the relevance of these “minor” mutations for their subsequent selection is currently uncertain and under investigation.

References


84. Zenz T, Hoth P, Busch R, et al. TP53 mutations and outcome after fludarabine and cyclophosphamide (FC) or FC plus rituximab (FCR) in the CLL8 Trial of the GCLLSG. Blood. 2009;114:1267a.


