Decitabine and Melphalan Fail to Reactivate p73 in p53 Deficient Myeloma Cells

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Abstract: (1) Background: TP53 deficiency remains a major adverse event in Multiple Myeloma (MM) despite therapeutic progresses. As it is not possible to target TP53 deficiency with pharmacological agents, we explored the possibility of activating another p53 family member, p73, which has not been well studied in myeloma. (2) Methods: Using human myeloma cell lines (HMCLs) with normal or abnormal TP53 status, we assessed TP73 methylation and expression. (3) Results: Using microarray data, we reported that TP73 is weakly expressed in 47 HMCLs and mostly in TP53 wild type (TP53wt) HMCLs (p = 0.0029). Q-RT-PCR assays showed that TP73 was expressed in 57% of TP53wt HMCLs (4 out of 7) and 11% of TP53 abnormal (TP53abn) HMCLs (2 out of 18) (p = 0.0463). We showed that TP73 is silenced by methylation in TP53abn HMCLs and that decitabine increased its expression, which, however, remained insufficient for significant protein expression. Alkylating drugs increased expression of TP73 only in TP53wt HMCLs but failed to synergize with decitabine in TP53abn HMCLs. (4) Conclusions: Decitabine and melphalan does not appear as a promising combination for inducing p73 and bypassing p53 deficiency in myeloma cells.

Keywords: multiple myeloma; p53; p73; CpG methylation; decitabine; alkylating drugs

1. Introduction

Multiple Myeloma (MM) is characterized by an important biological heterogeneity related to recurrent chromosomal abnormalities, which occurs early in the disease development at the MGUS (Monoclonal Gammapathy of Undetermined Significance) stage [1]. The recurrent chromosomal abnormalities are IgH gene translocation with recurrent partners located on chromosomes 4, 6, 11, 16 and 20 or hyperdiploidy of odd chromosomes [1]. Different prognoses and overall survivals are associated with this first heterogeneity. However, and independently from this heterogeneity, the most adverse prognosis is related to chromosomal deletion of TP53 [2]. Mutations of TP53 were exclusively found in myeloma cells displaying a semi-deletion of the short arm of chromosome 17 (del17p) [3]. The frequency of TP53 mutations, which is low at diagnosis (around 3%), increases with relapses and is high in plasma cell leukemia (PCL, >30%) and human myeloma cell lines (HMCLs, >70%), these latter mostly deriving from extramedullary MM and mainly from PCL [4–8]. Loss of function of mutant p53 is believed to be related to conformation deficiency: therefore, small molecules, such as
RITA and Prima-1<sup>Met</sup>, which were selected for their ability to induce cell death in p53 mutated cells were shown to bind to p53 protein [9]. However, in our hands, both molecules induced massive cell death but their efficacy was unrelated to TP53 status and p53 expression [10,11]. Because del(17)p and TP53 mutations, which are irreversible, are an adverse event whatever the treatment regimen, alternative treatments that bypass or circumvent p53 are needed. For instance, the p53 family member p73 could be of interest as p73 is also able to transactivate pro-apoptotic genes [12,13]. Moreover, TP73 is rarely mutated but frequently silenced by CpG methylation in hematological malignancies and in multiple myeloma (MM) [14–17]. TP73 expression might thus be reactivated by drugs preventing CpG methylation after DNA replication such as decitabine. To assess the interest in activating p73 in myeloma cells, we studied p73 expression and regulation, TP73 methylation and TP73 sequencing in a large panel of human myeloma cell lines HMCLs with a normal or abnormal TP53 status [7,18].

2. Results

2.1. TP73 Is Preferentially Constitutively Expressed in TP53<sup>wt</sup> HMCLs

We assessed expression of TP73 mRNA in 47 HMCLs using Affymetrix microarray data [7]. Although expression level was very low, HMCLs with normal TP53 status (TP53<sup>wt</sup>) appeared to significantly express higher level of TP73 mRNA than TP53<sup>abn</sup> HMCLs (p = 0.0029, Mann–Whitney test, Figure 1). To confirm this observation, we further selected 25 HMCLs displaying either a normal (n = 7) or an abnormal TP53 status (n = 18, 3 TP53 deleted (TP53<sup>del</sup>) and 15 TP53 mutated (TP53<sup>mut</sup>) HMCLs) (Table 1) [7,11,18]. Status of TP53 and TP73 was determined by sequencing of RT-PCR products and/or whole exon sequencing. Q-RT-PCR assays showed that TP73 was expressed in four out of seven TP53<sup>wt</sup> HMCLs (AMO1, MDN, MM1S, NAN11), in one out of three TP53<sup>del</sup> HMCLs (JJN3) and in one out of fifteen TP53<sup>mut</sup> HMCLs (XG11), p = 0.0463, Mann–Whitney test, (Figure 1B). p73 expression was confirmed at the protein level using Western blotting for MDN, MM1S, NAN11, JJN3 and XG11 but not for AMO1 (Figure 1C). Whole exon sequencing showed that only KMM1 and XG11 displayed a missense mutation (A211S and I626V, respectively). However, XG11, but not KMM1, expressed TP73 and p73. The strong p73 expression in XG11 might be related to the presence of the mutation within the transactivation inhibitory domain, which was reported to be involved in p73 degradation [19].

| HMCL | Name | Translocation | TP53 Status and Expression | TP73 Status and Expression |
|------|------|---------------|-----------------------------|-----------------------------|
| AMO1 | unknown | wt + | wt + | - |
| MDN | (11;14) | wt + | wt + | + |
| MM1S | (14;16) | wt + | wt + | - |
| NAN9 | (4;14) | wt + | wt + | - |
| NAN11 | (14;16) | wt + | wt + | - |
| XG6 | (16;22) | wt + | wt + | - |
| XG7 | (4;14) | wt + | wt + | - |
| JJN3 | (14;16) | deletion | wt + | - |
| KMM11 | (4;14) | deletion | wt + | - |
| NAN8 | (4;14) | disrupted | wt + | - |
| JIM3 | (4;14) | R275C | wt + | - |
| Karpass620 | (11;14) | C135Y | wt + | - |
| KMM1 | (6;14) | C135F | + | A211S + |
| XMS12PE | (11;14) | R337L | + | wt + |
| LP1 | (4;14) | E286K | + | wt + |
| L363 | (20;22) | S261T | + | wt + |
| NAM1 | (14;16) | E180KSTOP | + | wt + |
| NAM6 | (14;16) | Indel | - | wt + |
| NAM7 | (11;14) | Indel | - | wt + |
| OPM2 | (4;14) | R175H | + | wt + |
| SMKMM2 | (11;14) | K132N | + | wt + |
| XG11 | (11;14) | C135Y | - | wt + |
| XG1 | (11;14) | Y126N | + | wt + |
| XG2 | unknown | C176Y | + | wt + |
| XG5 | (11;14) | R282H | + | wt + |

nd: not done; 1 disrupted by amplification of exons 1, 2, 3, 4, 5 and 6; 2 lack of intron 7 splicing; 3 deletion of exons 7, 8 and 9; 4 deletion of exon 11; 5 truncated form.
transactivation, but rather to a weak transactivation. In parallel, we measured the expression level of TP73 (MS-PCR) targeting the CpG island upstream of the gene (Figure 2). PCR-1 was run on bisulfite converted DNA with primers that did not involve CpG. The PCR-1 product was then used for three nested PCRs; two, respectively, specific of unconverted CpG (methylated PCR, M-PCR) and converted CpG (unmethylated PCR, U-PCR) sequence; and one quantifying the PCR-1 product (PCR-Q, Figure 2).

To assess the methylation level in TP73, we performed nested methyl-specific PCR (MS-PCR) targeting the CpG island upstream of the gene (Figure 2). PCR-1 was run on bisulfite converted DNA with primers that did not involve CpG. The PCR-1 product was then used for three nested PCRs; two, respectively, specific of unconverted CpG (methylated PCR, M-PCR) and converted CpG (unmethylated PCR, U-PCR) sequence; and one quantifying the PCR-1 product (PCR-Q, Figure 2).

The data suggested that the lack of p73 detection by Western blotting was not related to the lack of TP73 expression (exons 7–10), the full-length TAp73 (exons 2–6) and the ΔNp73 (exons 3′–6) isoforms, respectively. The TAp73 isoform was increased in the three TP73mut HMCLs, although in KMS12PE the ΔNp73 isoform was the main increased isoform (Figure 3C). The data suggested that the lack of p73 detection by Western blotting was not related to the lack of TAp73 transactivation, but rather to a weak transactivation.

Figure 1. Constitutive TP73 expression was mainly restricted to TP53wt HMCLs: (A) Constitutive expression of TP73 in 47 HMCLs was performed by microarray (Affymetrix) and analyzed according to TP53 status. (B) Constitutive expression of TP73 was mainly restricted to TP53wt HMCLs. Expression was assessed by Q-PCR in 25 HMCLs. The histograms represent the mean ± SEM of 3 experiments (SEM fall within the symbols). *, p < 0.05. (C) Constitutive expression of p73 was mainly restricted to TP73wt HMCLs. Expression of p73 and p53 was assessed by Western blotting with anti-p73 and anti-p53 antibodies.

2.2. Decitabine Decreased TP73 Methylation and Induced TP73 Expression

To assess the methylation level in TP73 promoter, we performed nested methyl-specific PCR (MS-PCR) targeting the CpG island upstream of the gene (Figure 2). PCR-1 was run on bisulfite converted DNA with primers that did not involve CpG. The PCR-1 product was then used for three nested PCRs; two, respectively, specific of unconverted CpG (methylated PCR, M-PCR) and converted CpG (unmethylated PCR, U-PCR) sequence; and one quantifying the PCR-1 product (PCR-Q, Figure 2). PCRs were run in HMCLs treated or not with decitabine to assess the methylation status (Figure 3A). In parallel, we measured the expression level of TP73 by Q-PCR (Figure 3B). In JIM3, KMS12PE and XG5, TP73 was found methylated and decitabine induced a decrease in M-PCR and an increase in U-PCR, which correlated with an increase in TP73 expression. However, despite the increase in TP73 expression, we could not detect any protein. Because anti-p73 Ab was directed against the N terminal part of the protein (amino acids 1–62) and could thus not detect ΔNp73, we performed three RT-PCRs to assess the total TP73 expression (exons 7–10), the full-length TAp73 (exons 2–6) and the ΔNp73 (exons 3′–6) isoforms, respectively. The TAp73 isoform was increased in the three TP73mut HMCLs, although in KMS12PE the ΔNp73 isoform was the main increased isoform (Figure 3C). The data suggested that the lack of p73 detection by Western blotting was not related to the lack of TAp73 transactivation, but rather to a weak transactivation.
Figure 2. Targeting of CpG islands in TP73. Upper and lower sequences represent the native and bisulfite converted TP73 DNA sequence, respectively. The CpG are indicated with "+". Primers used for PCR1, M-PCR (Methylated-PCR), U-PCR (Unmethylated methylation) and PCR-Q (Quantifying PCR) are indicated by underlines. +1 indicates the first transcripted nucleotide.
Figure 3. Cisplatin or melphalan induced the expression of TAp73 isoform in TP53wt HMCLs but not in TP53mut HMCLs. (A) TP73 is mainly methylated in TP53mut HMCLs. MS-PCRs were performed after bisulfite conversion of DNA extracted from HMCLs treated or not with decitabine (5 µM, 72 h). M and U indicated methylated and unmethylated control DNAs. (B) Decitabine induced TP73 expression. TP73 expression was assessed by Q-PCR, as described in the legend of Figure 1. (C) Decitabine induced expression of several TP73 isoforms. RT-PCR expression of TAp73 isoform. RT-PCR expression of TAp73 and ΔNp73 was performed after a 16-h treatment with melphalan or cisplatin. TP53wt HMCLs were treated with 7 µM of melphalan or cisplatin, JJN3 cells were treated with 30 µM of melphalan or 20 µM of cisplatin. (E) Cisplatin or melphalan increased the expression p73 in TP53wt HMCLs. Western blots were performed after an overnight treatment with melphalan or cisplatin as described in (A). Right part represents the quantification of p73 and p53 over actin expression from two independent experiments (mean ± SEM). (F) Decitabine and melphalan or cisplatin failed to induce p73 expression in TP53mut HMCLs. HMCLs were treated 72 h with decitabine (5 µM) prior to an overnight treatment with 45 µM of melphalan or 55 µM of cisplatin. JJN3 proteins were loaded as a control of p73 expression.
2.3. Melphalan and Cisplatin Induced TP73 Expression in TP53<sup>wt</sup> HMCLs but Not in Decitabine-Treated TP53<sup>mut</sup> HMCLs

To further study TP73 regulation, we treated four HMCLs that constitutively expressed p73 with alkylating drugs, melphalan and cisplatin and determined by RT-PCR and Western blotting the regulation of TP73 and p73. Regulation of p53 expression was assessed as a control of drug response [20]. Alkylating drugs increased the expression of the p73 full-length isoform in the TP53<sup>wt</sup> MDN, MM1S and NAN11 HMCLs but not in TP53 deleted (TP53<sup>del</sup>) JJN3 HMCL (Figure 3D). MDN also expressed the ΔNp73 isoform that was slightly increased by the drugs. By Western blotting, we confirmed that melphalan and cisplatin similarly increased p73 expression in the three TP53<sup>wt</sup> HMCLs but failed to increase p73 expression in TP53<sup>del</sup> JJN3 (Figure 3E). As expected, p53 expression was increased by both drugs in the TP53<sup>wt</sup> MDN, MM1S and NAN11 HMCLs but not in TP53<sup>del</sup> JJN3 cells [20].

We then assessed whether melphalan could increase expression of p73 in decitabine-treated TP53<sup>mut</sup> HMCLs. We selected the three HMCLs that displayed a strong increase in TP73 expression upon decitabin treatment, i.e., JIM3, KMS12PE and XG5 (Figure 3B). As shown in Figure 3F, alkylating drugs failed to increase any p73 expression that remained undetectable in the 3 TP53<sup>mut</sup> HMCLs.

3. Discussion

In myeloma, regulation of p73 expression has not been deeply investigated yet. For instance, TP73 expression has been shown to be induced by PRIMA-1<sup>Met</sup> in several HMCLs including JJN3 in which p73 was reported to be partly involved in PRIMA-1<sup>Met</sup>-induced cell death [17,21]. However, we did not confirm these results in JJN3, suggesting that JJN3 might be misidentified between the different laboratories. In hematological malignancies, TP73 that does not display frequent mutations is known to be silenced by Cpg methylation [14]. Indeed, whole exon sequencing showed that only two HMCLs (KMM1 and XG11) harbored a mutation in TP73 gene. We thus assessed TP73 methylation and regulation in a large number of HMCLs. Our results showed that TP73 is mainly silenced by methylation and that the methylation inhibitor decitabine reversed the methylation-mediated silencing. However, the TP73 expression remained too weak for allowing a detectable p73 expression by Western blotting. We showed that decitabine-induced expression of both TAp73 and ΔNp73 isoforms: ΔNp73, which is lacking the transactivation domain, is known to inhibit TAp73 binding to DNA [19]. In KMS12PE, the high decitabine-induced expression of ΔNp73 isoform might explain the lack of p73 detection by Western blotting because the anti-p73 Abs was directed against the N terminal part of the protein (aa 1-62). TP73 gene also harbors an intrinsic Cpg island that governs ΔNp73 isoform transcription. Thus, TP73 gene appears particularly methylated in MM cells and demethylation might equally induce expression of TAp73 and ΔNp73 isoforms, the latter being an inhibitory isoform [19]. We found that p73 was constitutively expressed in six HMCLs: MDN, MM1S, NANN1 and XG10 expressed a wildtype p53 protein. JJN3 lacked p53 expression and XG11 expressed a mutant p53 protein. Of note, p73 was overexpressed in XG11 despite a moderate TP73 expression suggesting a stabilization/lack of degradation of the protein that might be related to the presence of the mutation in the transactivation inhibitory domain, which is involved in degradation [22]. In three out of four HMCLs that constitutively expressed p73 and displayed an unmethylated profile of Cpg, alkylating drugs increased both TP73 and p73 expression: this regulation seemed to be restricted to TP53<sup>wt</sup> HMCLs as it did not occur in the TP53<sup>del</sup> JJN3 HMCL. This finding is consistent with the possible regulation of TP73 expression via p53/p21/E2F1, which can only occur in TP53<sup>wt</sup> HMCLs [23]. Indeed, alkylating drugs were unable to increase p73 expression in the three TP53<sup>mut</sup> HMCLs that displayed a TP73 expression after decitabine treatment. Moreover, MDM2, which stabilizes p73 instead of inducing its degradation [24], could take a part in the p73 increase induced by alkylating drugs, because MDM2 is a p53 target gene mostly expressed in TP53<sup>wt</sup> HMCLs [18]. Nevertheless, the role of p53 in melphalan-induced p73 regulation, if any, requires further investigations. On the other hand, TP73 expression was shown to be regulated by HDAC: indeed, the HDAC inhibitor sodium butyrate...
increased the “free” E2F1 pool and therefore TP73 transactivation independently from p53 [25]. We did not investigate the HDAC-mediated regulation of p73 in myeloma cells but it would be of interest to study HDAC inhibitors in combination with decitabine in p53 deficient cells. In summary, our data show that decitabine and melphalan does not appear as a promising combination for bypassing p53 deficiency in myeloma cells.

4. Materials and Methods

4.1. HMCLs and Reagents

HMCLs were previously described [7,11]. Unmethylated/methylated control DNAs, and the bisulfite conversion kit were purchased from Active Motif (La Hulpe, Belgium). Anti-p73 A300-126A and anti-p53 DO-1 Abs were purchased from Bethyl Laboratories (Euromedex, Souffelweyersheim, France) and Millipore (Saint-Quentin en Yvelines, France), respectively. Anti-actin was purchased from Millipore. Cisplatin and melphalan were purchased from Sigma Aldrich (Saint-Quentin-Fallavier, France). Quantitative PCR probes were purchased from Taqman (Thermofisher, Saint-Herblain, France). Quantitative RT-PCR assays were performed as previously described [7].

4.2. Bisulfite Treatment, Methylation Specific PCRs and RT-PCR

DNAs were treated with bisulfite as indicated by the supplier. Methylation specific PCRs (M-PCR, U-PCR) and quantifying PCR (PCR-Q) were performed after a first amplification (PCR1). PCR1, M-PCR, U-PCR and PCR Q were run with 25, 17, 20 and 19 cycles, respectively. The following primers were used: PCR-1 GTTTTGGGTTTTGGGAGTTGAGAG and ACCACCCACTTCTTCTATATAAA (874 bp); M-PCR GGGTTATTATGGGTAGAGGATATC and ACATACTAAACCGAATCCCGACCTC (109 bp); U-PCR GGGTTATTATGGGTAGAGGAGATATT and ACATACTAAACAAATTTCAACACACTCTC (112 bp); and PCR Q TAAATAGTGGTGAGTTATGAAGATGT and TACACCAACCCCTAACTAAAAACC (285 bp). Extracted RNAs were reversed transcripted and amplified as previously described [12].

For TP73 RT-PCR assays, the following primers were used: TAp73 ex2-6 CACCACGTTTGAGCACCCTCT and AGATTATTGCTTCCACGGCG (630 bp); TP73 ex7-10 GACCCAATTACACCACCACCTC and CCAGGCTCTTCTTCCAGCTTC (389 bp); and ΔNp73 ex3′-6 CCATGCTGTACGTCGGTGAC and CCAAATCCTTCTCCCTATCC (519 bp). For TP73 Q-PCR assays, the TP73 (Hs01056230_m1) and RPL37A (Hs01102345_m1) probes were used.

4.3. Western Blotting

Expression of p53 and p73 was determined by Western blotting as previously described [11]. A minimum of 80 µg of proteins was loaded in each lane.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

| Abbreviation | Description                     |
|--------------|---------------------------------|
| HMCL         | Human Myeloma Cell Line         |
| MM           | Multiple Myeloma                |
| MS-PCR       | Methylation-specific PCR        |
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