p53 Mutations in Cyclophosphamide-associated Bladder Cancer


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Abstract

Cyclophosphamide is a known bladder carcinogen, with cumulative dose directly related to increased risk. There is no consensus, however, on which major cyclophosphamide metabolite (i.e., acrolein or phosphoramide mustard) drives bladder carcinogenesis. We examined 19 cyclophosphamide-related bladder tumors to test the hypothesis that they might contain somatic mutations in the p53 tumor suppressor gene that could link a specific metabolite to the etiology of these cancers. Forty-three % (9 of 19) of the cases had a mutation in p53, with a predominance at G:C bp (7 of 9, 77%), a preference for non-CpG sites (6 of 7, 86%), and frequent G:C→A:T transitions (5 of 7, 71%). The p53 mutation spectrum of these cyclophosphamide-associated bladder cancers differed significantly from patterns reported for sporadic (P = 0.020), smoking-related (0.043), and schistosomiasis-linked (P = 0.002) tumors but not arylamine-associated neoplasms (P = 0.860). Differences between the cyclophosphamide and arylamine-associated spectra included an unusual degree of clustering of exon 6 mutations (43% versus 17%, respectively) and an absence of multiple mutations in the former. Notably lacking in our series were G:C→T:A transversions, the principal mutation associated with acrolein. Instead, the mutation spectrum matches the phosphoramide mustard addition sequences determined by a repetitive primer-extension assay (P = 0.024), indicating that this metabolite might be a key mutagen in cyclophosphamide-related bladder cancer.

Introduction

Cyclophosphamide is a known bladder carcinogen with a highly significant relationship between cumulative dose and bladder cancer risk (1). This cytotoxic agent is prescribed to 500,000 patients each year worldwide (2), and the resulting bladder cancers may have both early onset and an aggressive course (3). Although three major, DNA-binding metabolites (phosphoramide mustard, nornitrogen mustard, and acrolein; Fig. 1A) result from the metabolism of cyclophosphamide by mixed function oxidases, there is no consensus on which product drives bladder carcinogenesis. Many investigators consider the primary mutagen to be phosphoramide mustard (4–6), which forms monofunctional and bifunctional guanine adducts (7); nornitrogen mustard may also play a role because it has similar chemical properties (Ref. 7; Fig. 1B). Acrolein is mutagenic in animal models (4, 5, 7, 8) and human cells in vitro (9, 10), but it is not a proven carcinogen, possibly because of its extreme toxicity (11). Clinical data correlating acrolein-induced hemorrhagic cystitis with subsequent bladder cancer is conflicting (12–14). Given the uncertainty over the role of acrolein as a bladder carcinogen, we evaluated a series of bladder cancers which followed cyclophosphamide therapy (1) to test the hypothesis that patterns of somatic mutations might link acrolein, phosphoramide mustard, or nornitrogen mustard to the etiology of these cancers. We tested for mutations in the p53 tumor suppressor gene because it plays a prominent role in the development of bladder cancer (reviewed in Ref. 15) and because characteristic mutational spectra have been reported for two other bladder carcinogens, tobacco (16) and schistosomiasis (17).

Materials and Methods

Study Population. All available tissue samples were obtained from a case-control study of secondary bladder cancer (1) conducted within a cohort of 6171 2-year survivors of non-Hodgkin’s lymphoma (18). In this analytic investigation, which represents the largest study to date of cyclophosphamide-associated bladder cancer, the relative risk associated with cumulative doses of cyclophosphamide <20, 20–50, and >50 g were 2.4, 6.3, and 14.5, respectively (P-trend = 0.004; Ref. 1). With the approval of local boards governing research on human subjects, resection or biopsy tissues were requested for all patients in the case-control study, with specimens available for 19 primary bladder cancers from 18 patients. The second bladder cancers occurred an average of 12 years after lymphoma diagnosis. Data on cumulative cyclophosphamide dose, radiation dose to bladder, interval between lymphoma and bladder cancer, and other pertinent clinical information for the 18 patients are summarized in Table 1. All subjects were treated...
with cyclophosphamide (median cumulative dose, 16.6 g; range, 6–125.2 g), and some also received radiotherapy.

Genomic DNA Extraction, p53 PCR Amplification, and DNA Sequence Analysis. Tumor and nontumor tissues were microdissected from unstained, paraffin-embedded, formalin-fixed histological sections. After proteinase K digestion, genomic DNA was isolated by organic extraction as described (19). Exons 5–8 were amplified individually using nested primers, and each product was sequenced as reported previously (19); mutations were confirmed by finding the same base change in two independent PCR products.

Statistical Analyses. The overall type and distribution of mutations among the 570 bp in exons 5–8 of the p53 gene in the cyclophosphamide-associated bladder cancers were compared with the mutational spectra observed in sporadic bladder cancers and those linked to tobacco use, schistosomiasis, and arylamine exposure using the procedure described by Cariello et al. (20). This method uses the Adams and Skopek algorithm to estimate P corresponding to an exact multivariate hypergeometric test for contingency tables (21). A random number generator simulates a large number of spectra based on the multivariate hypergeometric distribution, conditional on the numbers and locations of mutations in the two series to be compared.

The frequency of specific mutations within p53 exons was compared with the distribution reported for bladder cancers following exposure to other known carcinogens or to sporadic
<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Tobacco use</th>
<th>NHL DX (mo/yr)</th>
<th>NHL stage</th>
<th>All therapy</th>
<th>Begin-End (mo/yr)</th>
<th>Total dose of CTX (g)</th>
<th>Total radiation dose to bladder (Gy)</th>
<th>Interval NHL to bladder cancer (yr)</th>
<th>NHL to bladder cancer</th>
<th>p53 sequence analysis</th>
<th>Codon/Mutation</th>
<th>Base change</th>
<th>CpG site</th>
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<tbody>
<tr>
<td>1</td>
<td>67/M</td>
<td>Ex-smoker</td>
<td>1/74</td>
<td>Unk</td>
<td>CVP</td>
<td>1/78-12/78</td>
<td>11/77-12/77</td>
<td>24.0</td>
<td>34.7</td>
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<td>WT</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2</td>
<td>62/M</td>
<td>5 cigars/day</td>
<td>11/78</td>
<td>II</td>
<td>RT</td>
<td>1/78-12/78</td>
<td>1/79-1/79</td>
<td>13.5</td>
<td>33.8</td>
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<td>3</td>
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<td>II</td>
<td>RT</td>
<td>7/77-8/77</td>
<td>1/78-12/77</td>
<td>8.8</td>
<td>&lt;0.1</td>
<td>10.0</td>
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<td></td>
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<td>4</td>
<td>64/F</td>
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<td>3/69</td>
<td>III</td>
<td>RT</td>
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<td>CVP</td>
<td>2/75-7/75</td>
<td>7/75</td>
<td>10.3</td>
<td>19.5</td>
<td>6.1</td>
<td>1/75-2/75</td>
<td>WT</td>
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<tr>
<td>6</td>
<td>61/M</td>
<td>&gt;2 PPD × 40 yr</td>
<td>12/70</td>
<td>III</td>
<td>RT</td>
<td>12/75-1/76</td>
<td>1/75-1/75</td>
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<td>7</td>
<td>69/M</td>
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<td>7/78</td>
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<td>RT</td>
<td>1/72-3/77</td>
<td>3/77-4/77</td>
<td>13.3</td>
<td>&lt;0.1</td>
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<tr>
<td>8</td>
<td>68/M</td>
<td>Ex-smoker, other tobacco</td>
<td>10/78</td>
<td>Unk</td>
<td>CTX</td>
<td>1/79-1/79</td>
<td>11/78-4/79</td>
<td>20.2</td>
<td>&lt;0.1</td>
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<tr>
<td>9</td>
<td>65/M</td>
<td>Ex-smoker 1–2 PPD</td>
<td>3/78</td>
<td>I</td>
<td>COPP</td>
<td>6/78-12/78</td>
<td>4/78-5/80</td>
<td>6.0</td>
<td>38.8</td>
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<td>I</td>
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<td>4/74-5/74</td>
<td>12.0</td>
<td>23.8</td>
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<tr>
<td>12</td>
<td>61/M</td>
<td>Ex-pipe</td>
<td>7/84</td>
<td>IV</td>
<td>RT</td>
<td>7/84-1/85</td>
<td>7/84-1/85</td>
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<td>0.9</td>
<td>3.4</td>
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<tr>
<td>14</td>
<td>56/F</td>
<td>1–2 PPD</td>
<td>4/81</td>
<td>IV</td>
<td>CHOP</td>
<td>5/81-11/82</td>
<td>5/81-11/82</td>
<td>18.0</td>
<td>8.7</td>
<td>10.8</td>
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<td>WT</td>
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<tr>
<td>15</td>
<td>38/M</td>
<td>1–2 PPD</td>
<td>12/78</td>
<td>IV</td>
<td>CTX</td>
<td>9/79-9/82</td>
<td>9/79-9/82</td>
<td>125.2</td>
<td>0.9</td>
<td>3.4</td>
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<tr>
<td>16</td>
<td>52/M</td>
<td>2 PPD × 36 yr</td>
<td>10/83</td>
<td>II or III</td>
<td>CHOP</td>
<td>11/83-3/84</td>
<td>11/83-3/84</td>
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<tr>
<td>17</td>
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<td>CHOP</td>
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<td>7/81-12/81</td>
<td>6.4</td>
<td>(None)</td>
<td>5.7</td>
<td>7/81-12/81</td>
<td>WT</td>
<td></td>
<td></td>
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</table>

**Notes:**
- *a* At diagnosis of non-Hodgkin's lymphoma.
- *b* All histories refer to cigarette use unless noted otherwise.
- *c* DX, diagnosis; ABVD, doxorubicin, bleomycin, vincristine, and dacarbazine; BACOP, cyclophosphamide, bleomycin, doxorubicin, vincristine, and prednisone; CDDP, cisplatin; CHLB, chlorambucil; CHOP, cyclophosphamide, doxorubicin, vincristine, and prednisone; CTX, cyclophosphamide, vincristine, procarbazine, and prednisone; CVP, cyclophosphamide, vincristine, and prednisone; IMTX, intrathecal methotrexate; MEV, cyclophosphamide, methotrexate, and vincristine; NA, not applicable; NHL, non-Hodgkin's lymphoma; Pap, TCC, papillary transitional cell carcinoma; PPD, packs/day; RT, radiotherapy; SqCC, squamous cell carcinoma; TCC, transitional cell carcinoma; Unk, unknown; WT, wild type.
- *d* Cumulative dose of cyclophosphamide.
- The mutation in Patient 10 occurred in the 5' splice site for exon 8 adjacent to the last nucleotide of codon 261.
- Case was not included in the prior analytic investigation (1) because strict eligibility criteria were not met or because the bladder cancer developed after the close of follow-up (December 31, 1989).
- *e* Patient 16 had a second bladder cancer diagnosed 9.6 years after the NHL.

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cases by means of the two-tailed Fisher's exact test. An exact permutation test for trend (21) was conducted to test for a dose-response between cumulative amount of cyclophosphamide and p53 mutations. Exact binomial Ps were calculated to test whether a significant excess of mutations or adducts occurred within a selected class of nucleotides.

Results

p53 Mutations. Nine p53 mutations occurred in 8 of 18 patients with secondary bladder cancer. Patient 16 had two bladder cancers that were diagnosed 3 years apart and contained different p53 mutations; due to the time interval, disparate cancers that were diagnosed 3 years apart and contained dose-response between cumulative amount of cyclophosphamide and p53 mutations. Exact binomial Ps were calculated to test for a significant excess of mutations or adducts occurring within a selected class of nucleotides.

<table>
<thead>
<tr>
<th>Exposure before bladder cancer</th>
<th>Mut. Freq. (%)</th>
<th>n</th>
<th>P</th>
<th>G:C→A:T (CpG)</th>
<th>A:T→G:C (non-CpG)</th>
<th>Del/Ins/Other</th>
<th>Mutations in exon 6 %</th>
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<tbody>
<tr>
<td>Cyclophosphamide</td>
<td>49</td>
<td>9</td>
<td></td>
<td>0 5 0 2</td>
<td>1 1 0 0</td>
<td></td>
<td>44 NA</td>
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<tr>
<td>Arylamine</td>
<td>47</td>
<td>27</td>
<td>0.860</td>
<td>0 17 3 1</td>
<td>4 1 0 0</td>
<td></td>
<td>17 0.300</td>
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<tr>
<td>Schistosomiasis</td>
<td>40</td>
<td>71</td>
<td>0.002</td>
<td>21 20 7 9</td>
<td>6 2 0 0</td>
<td></td>
<td>10 0.045</td>
</tr>
<tr>
<td>Tobacco</td>
<td>NA</td>
<td>81</td>
<td>0.043</td>
<td>9 32 9 14</td>
<td>3 1 5 0</td>
<td></td>
<td>7 0.044</td>
</tr>
<tr>
<td>Sporadic</td>
<td>34</td>
<td>113</td>
<td>0.020</td>
<td>18 24 16 18</td>
<td>11 5 5 0</td>
<td></td>
<td>6 0.012</td>
</tr>
</tbody>
</table>

* Mut. Freq., mutation frequency; NA, not applicable.
* Comparison of the overall p53 mutation spectrum in cyclophosphamide-associated tumors with those observed in the indicated series. Data for sporadic, schistosomiasis, tobacco, and arylamine-associated bladder cancers were collected from the p53 mutation database (54) and compared with the cyclophosphamide spectrum by Monte Carlo analysis (Ref. 20; see "Materials and Methods" for additional description). Statistically significant (P < 0.05) differences were observed between the pattern of p53 mutations in cyclophosphamide-associated cases compared with sporadic, schistosomiasis, and tobacco-related tumors.

The iI 3 mutations from "sporadic" tumors exclude all other groups. The 113 mutations from "sporadic" tumors exclude all other groups.

The Cyclophosphamide Mutation Spectrum Is Distinct among Bladder Cancers and Concentrates in Exon 6. The overall p53 mutation spectrum in cyclophosphamide-associated tumors differed significantly from those described in sporadic (P = 0.020), smoking-related (P = 0.043), and schistosomiasis-associated (P = 0.002) bladder cancer but not arylamine-linked cancers (P = 0.86). The predominant mutations in our series were G:C→A:T transitions occurring only at non-CpG sites (five of five), similar to mutations described for arylamine. In contrast, ratios of G:C→A:T transitions at non-CpG versus CpG sites for most other bladder cancer series varied from 1:1 (schistosomiasis) to 1:4 (tobacco).

Cyclophosphamide-associated bladder cancers differed markedly from those related to arylamine in the degree of mutational clustering in exon 6 and in the absence of multiple lesions. Four (44%) of the nine mutations in our study and three (43%) of the seven mutations at G:C bp occurred in exon 6, a disproportionate representation, given the small size of this exon (37 codons) compared with exons 5, 7, and 8 (142 codons). Moreover, the percentage of mutations in exon 6 in cyclophosphamide-associated tumors was considerably larger than the frequency (6–17%) reported for sporadic bladder cancers (P = 0.012) or those linked to tobacco (P = 0.044), schistosomiasis (0.045), or arylamine (P = 0.30, see Table 2). Multiple p53 mutations, which were not observed in our series, were prominent in bladder malignancies after arylamine exposure (24).

Investigation of a Possible Dose-Response Relationship between Cyclophosphamide and p53 Mutation Rates. Mutations at G:C bp in non-CpG sites (n = 6) were categorized according to cyclophosphamide dose groups used in the analysis of the prior case-control study (1); 27% (3 of 11) and 50% (1 of 2) occurred in the <20-, 20–50-, and >50-g groups, respectively (P = 0.40). A similar pattern was observed when all p53 mutations were analyzed according to cumulative cyclophosphamide dose categories (P = 0.31).

Discussion

Cyclophosphamide Produces a Mutation Spectrum That Matches Phosphoramide Mustard Adduction Sites. We provide the first description of p53 mutations in cyclophosphamide-related bladder cancers. The p53 mutation spectrum in these tumors is distinguished by a predominance of mutations at G:C bp, a preference for non-CpG sites, an excess of G:C→A:T transitions, and a clustering in exon 6. This unusual constellation of features differs significantly from sporadic, smoking-related, and schistosomiasis-linked bladder cancers, and the concentration of mutations in exon 6 differs markedly from bladder tumors in these series (Table 2). The character and location of mutations in cyclophosphamide-related bladder cancer match the distribution of phosphoramide mustard adducts determined by a repetitive primer-extension assay (25). Both the mutations and the adducts have strong preferences for G:C bp at non-CpG dinucleotides, with significant excesses of p53 mutations and strong adduct sites in exon 6. Furthermore, three (43%) of seven mutations at G:C bp in our series occupied strong adduct sites. It is unlikely that this distribution occurred by chance alone (P = 0.018, binomial probability), because only 6.8% of G:C pairs are found at strong adduct sites. It is unlikely that this distribution occurred by chance alone (P = 0.018, binomial probability), because only 6.8% of G:C pairs are found at these locations. Taken together, our data suggest that phosphoramide mustard may be an important mutagenic metabolite in cyclophosphamide-related bladder cancer.

Phosphoramide Mustard Forms Guanine Adducts within Consensus Sequences. Phosphoramide mustard and nitroguanidino mustard form adducts predominantly at the N7-position of guanine, occasionally at the N3-position of guanine (reviewed in Refs. 5, 7, and 8), and rarely at adenine or thymidine (26, 27).
Consensus sequences flanking preferential guanine adduction sites were recently defined by a repetitive primer extension assay (25) used to map the location and intensity of DNA adducts in human cells. These experiments found phosphoramide mustard adducts only at guanine residues, as reported previously (26, 27). There were variations in binding intensity among guanines, and consensus sequences for strong, intermediate, and weak adduction sites were defined; it is notable that adduction of guanines flanked by cytosine was rare (25). Because acrolein adducts are not detected by this assay (25), the adducts were produced either by phosphoramide mustard or nornitrogen mustard. When these consensus sequences are compared with p53 coding regions, there is a striking clustering of strong adduction sites in exon 6 (n = 9) compared with exon 8 (n = 6) or exons 5 and 7 (n = 3 each; Fig. 2). It is unlikely that 9 (43%) of 21 strong adduct sites occurred in exon 6 by chance (P = 0.024, binomial probability), because this exon contains only 19.4% of G:C sites. Nornitrogen mustard may also be a plausible mutagen, but we cannot assess the contribution of this metabolite until its associated adduct distribution has been determined.

**Phosphoramide Mustard Adducts Match Cyclophosphamide-induced p53 Mutations.** All major features of the phosphoramide mustard adduct distribution are seen in the p53 mutations, including a strong preference for non-CpG sites and a notable exon 6 clustering. In fact, cogent arguments link all seven mutations at G:C bp in our series to phosphoramide mustard adducts. For example, all matched strong (3), intermediate (1), or weak (3) consensus adduction sequences and all but two of the mutation sites had at least 80% homology to the consensus adduction sequences (Ref. 25; Table 3). Five of seven mutations at G:C bp have a coding strand bias, which is characteristic of chemical carcinogens due to preferential repair of the transcribed strand (28, 29); it is notable that the two exceptions (i.e., patients 5 and 16) occupy strong adduction sites that may compensate for the rapid repair of the transcribed strand. Data for DNA repair rates within the p53 coding sequences are incomplete, but three of the mutations (i.e., patients 6, 13, and 16) occupy known slow spots for DNA repair, which is a predisposing factor for mutation (30).

**DNA-adduct hotspots were previously linked with characteristic mutations in a report correlating the distribution of p53 mutations in human lung cancer with the DNA adduct distribution of benzo(a)pyrene diol-epoxide, a carcinogen in tobacco smoke (31).** Our study provides the first example describing homology of carcinogen-DNA adduct hotspots with the pattern of p53 mutations in an iatrogenic cancer. There are no reports, to our knowledge, of the mutation spectrum generated by cyclophosphamide in animal or in vitro models.

**Acrolein: Toxicity, Adducts, and Mutation Spectrum.** Despite the established role of acrolein in hemorrhagic cystitis (14), there is insufficient evidence to classify it as a carcinogen (11). Three extended studies in animals have been negative (32, 33), including a 2-year ingestion study in rats (34). Although a recent investigation found initiating activity for bladder cancer when acrolein was administered intraperitoneally (35), complete carcinogenic activity could not be evaluated due to its extreme toxicity. Acrolein is considered mutagenic (9, 36–38), presumably due to the formation of DNA adducts; in aqueous solution, acrolein reacts with deoxyguanosine to produce two major isomers of the hydroxy-1,N2-propanodeoxyguanosine adduct and the 7,8-cyclic guanine adduct (Refs. 39–41; Fig. 1B). Because of the toxicity of acrolein (42), investigators have incorporated 1,N2-propanodeoxyguanosine into shuttle vectors.

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1, N2-Propanodeoxyguanosine has been used as a model because the hydroxy-1, N2-propanodeoxyguanosine adduct is unstable under conditions used for oligodeoxynucleotide synthesis.
Table 3  Correlation of mutations, adduct sites, and DNA repair rates

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<th>Patient no.</th>
<th>5′-3′ DNA sequence of Codon</th>
<th>Intensity</th>
<th>Phosphoramide mustard adducts</th>
<th>DNA repair rate</th>
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<td>18</td>
<td>5 GTG</td>
<td>Weak</td>
<td>tiGec3</td>
<td>-</td>
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<tr>
<td>16</td>
<td>6 GTG</td>
<td>Weak</td>
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</tr>
<tr>
<td>13</td>
<td>6 ACT</td>
<td>No</td>
<td>4</td>
<td>-</td>
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