DNA repair-deficient \textit{Xpa/p53} knockout mice are sensitive to the non-genotoxic carcinogen cyclosporine A: escape of initiated cells from immunosurveillance?

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The DNA repair-deficient \textit{Xpa} \textsuperscript{-/-} \textit{p53} \textsuperscript{+/+} (\textit{Xpa/p53}) mouse is a potent model for carcinogenicity testing, representing increased sensitivity toward genotoxic but surprisingly also toward true human non-genotoxic carcinogens. The mechanism of this increased sensitivity in \textit{Xpa/p53} mice toward non-genotoxic carcinogens is still unknown. Here, we investigated the mechanism of the human non-genotoxic carcinogen cyclosporine A (CsA) in the \textit{Xpa/p53} mouse model. \textit{Xpa/p53} mice exposed to CsA for 39 weeks showed a significantly increased lymphoma incidence as compared with untreated \textit{Xpa/p53} mice and CsA-treated wild-type (WT) mice. We excluded concealed genotoxicity of CsA in \textit{Xpa/p53} mice by mutant frequency analyses. As a next step, we used a genetic approach: immunodeficient DNA-PKcs mice, defective in the catalytic subunit of the DNA-dependent protein kinase, were crossed with \textit{Xpa} and \textit{Xpa/p53} mice. \textit{Xpa/p53} mice had an increased lymphoma incidence with shorter latency times as compared with DNA-PKcs-deficient WT and \textit{Xpa} mice. Surprisingly, also six of 15 DNA-PKcs/\textit{Xpa/p53} females had developed an adenocarcinoma of the mammary gland. Tumor responses in CsA-treated and DNA-PKcs-deficient \textit{Xpa/p53} mice were comparable as both genotypes developed mainly splenic lymphomas enriched in B lymphocytes. From our present studies, we hypothesize that levels of initiated precancerous cells are elevated in \textit{Xpa/p53} mice. These cells are insufficiently eliminated due to either suppression of the immune system by CsA or through immune-related DNA-PKcs deficiency. Based on the current studies and those conducted previously, we conclude that the \textit{Xpa/p53} model is an excellent adjunct to the current chronic rodent bioassay.

Introduction

The carcinogenic potency of compounds is traditionally tested in the classical chronic rodent bioassay. Since the early 1990s, transgenic mice have been introduced as adjunctive models for testing carcinogenicity because of their increased sensitivity to carcinogens (1). One of the transgenic mouse models with predictive value regarding carcinogenicity because of their increased sensitivity to carcinogens is still unknown. Here, we investigated the mechanism of the human non-genotoxic carcinogen cyclosporine A (CsA) in the \textit{Xpa/p53} mouse model. \textit{Xpa/p53} mice exposed to CsA for 39 weeks showed a significantly increased lymphoma incidence as compared with untreated \textit{Xpa/p53} mice and CsA-treated wild-type (WT) mice. We excluded concealed genotoxicity of CsA in \textit{Xpa/p53} mice by mutant frequency analyses. As a next step, we used a genetic approach: immunodeficient DNA-PKcs mice, defective in the catalytic subunit of the DNA-dependent protein kinase, were crossed with \textit{Xpa} and \textit{Xpa/p53} mice. \textit{Xpa/p53} mice had an increased lymphoma incidence with shorter latency times as compared with DNA-PKcs-deficient WT and \textit{Xpa} mice. Surprisingly, also six of 15 DNA-PKcs/\textit{Xpa/p53} females had developed an adenocarcinoma of the mammary gland. Tumor responses in CsA-treated and DNA-PKcs-deficient \textit{Xpa/p53} mice were comparable as both genotypes developed mainly splenic lymphomas enriched in B lymphocytes. From our present studies, we hypothesize that levels of initiated precancerous cells are elevated in \textit{Xpa/p53} mice. These cells are insufficiently eliminated due to either suppression of the immune system by CsA or through immune-related DNA-PKcs deficiency. Based on the current studies and those conducted previously, we conclude that the \textit{Xpa/p53} model is an excellent adjunct to the current chronic rodent bioassay.

Abbreviations: CsA, cyclosporine A; FACS, fluorescence-activated cell sorting; MLN, mesenteric lymph node; DNA-PKcs, catalytic subunit of the DNA-dependent protein kinase; WT, wild-type.

The dependence of cancer formation, either induced or spontaneously, and immunosurveillance failure has been shown before in several immunodeficient mouse models (14,15). One of these immunodeficient models is the interferon-\gamma receptor-deficient mouse, which shows an enhanced tumor development when bred in a p53-deficient background (16), a situation as found in \textit{Xpa/p53} mice. A more recent theory as to how CsA induces cancer is through a pathway that is mediated by transforming growth factor-\beta. Hojo et al. (17) found that CsA by itself alters the characteristics of cancerous cells by inducing the synthesis of transforming growth factor-\beta, a growth factor that promotes tumor cell invasion and metastasis. Furthermore, the authors showed that immunodeficient \textit{Scid} mice inoculated with tumor cells had an increased tumor incidence and more metastases when treated with CsA, compared with unexposed \textit{Scid} mice. This finding indicated that tumor induction by CsA might occur through another molecular pathway. This idea is supported by studies of other research groups (18,19), indicating that, in \textit{Xpa/p53} mice, this mechanism might also play an important role in tumor development initiated by CsA.

As the \textit{Xpa/p53} mouse is responsive to carcinogens with various modes of action, it is important to study the individual mechanisms of tumor development in this model. As such, we studied the effect of CsA in \textit{Xpa/p53} mice in more detail. To exclude that CsA carries concealed genotoxic activity, we performed gene mutation analyses in DNA repair-deficient \textit{Xpa/p53} mice. Furthermore, immunodeficient DNA-PKcs mice, defective in the catalytic subunit of the DNA-dependent protein kinase (DNA-PK), were used to solely study the effect of immunodeficiency on lymphoma induction. Lymphomas were characterized on T and B cell composition. We show that the non-genotoxic carcinogen CsA induces lymphomas with high frequency in \textit{Xpa/p53} mice by suppression of the immune system in these mice.

Materials and methods

Chemicals

Feed containing CsA at various concentrations was purchased from Altromin, Lage, Germany. CsA was kindly provided by Novartis (Basel, Switzerland). The following antibodies were used for immunohistochemical staining: CD3
hamster anti-mouse, CD19 rat anti-mouse and mouse anti-rat (BD Biosciences, Franklin Lakes, NJ), CD4 rat anti-mouse (La Roche, Basel, Switzerland), IgM rat anti-mouse (Serotech, Oxford, UK), rabbit anti-hamster peroxidase and rabbit anti-rat (Jackson, Suffolk, UK) and swine anti-rabbit peroxidase (DAKO, Carpinteria, CA). RPMI medium for fluorescence-activated cell sorting (FACS) analysis was obtained from Invitrogen (Breda, the Netherlands). Fluorescin isothiocyanate-, phycoerythrin-, PerCP-conjugated monoclonal antibodies for FACS analyses and FACS lysing solution were obtained from BD Biosciences Pharmingen.

**Mice**

All mice used in this study were in a congenic C57BL/6 background. Xpa−/− mice (3) were crossed with Xpa/p53−/− mice (20). Immunodeficient mice were obtained by crossing WT, Xpa−/−, Xpa+/−, and Xpa+/+ mice with DNA-PKcs knockout mice (21). To confirm genotypes, polymerase chain reaction analyses were performed as described previously (22). All mice were housed specific pathogen free in a climate-controlled room with a 12 h on/off light cycle. Feed and water were available ad libitum throughout the course of all experiments. The study was agreed upon by the institute’s Ethical Committee on Experimental Animals, in accordance to national legislation.

**Short-term carcinogenicity study**

The first of two short-term carcinogenicity studies were described previously by van Kreijl et al. (5). In short, groups of 15 male and 15 female WT, Xpa−/−, and Xpa+/− mice were fed diets containing 0, 18.7, 62.5, 190 or 500 p.p.m. CsA for 39 weeks. In addition, a positive control group was included, involving all three genotypes. These mice were fed 300 p.p.m. 2-acetylaminofluorene. To confirm previous findings, we repeated part of the study using groups of 10 male and 10 female Xpa+/− mice that were fed 500 p.p.m. CsA for 39 weeks. Parameters monitored included clinical signs, body weight, food intake, organ weights and gross and macroscopic pathology. At autopsy, major organs were isolated from each animal including spleen, mesenteric lymph node (MLNs), thymus, liver, kidneys, adrenals, brain, heart and testes, together with tumors and tissues with gross lesions. Of all tissues and tumors, part was preserved in formaldehyde. Subsequently, samples were embedded in paraffin wax, cut into 5 μm sections and stained with hematoxylin and eosin for histopathology.

**LacZ mutation analysis**

Six- to nine-week old WT, Xpa−/−, and Xpa+/− mice were treated with 500 p.p.m. CsA mixed in the feed for 12 weeks or control diet for 6 weeks. Each group consisted of three male and three female mice. As a positive control, mutant frequencies were analyzed in the liver of 300 p.p.m. 2-acetylaminofluorene-treated Xpa+/− mice. Total genomic DNA was isolated from spleen, MLNs and liver as described previously (23). Mutant frequencies were determined after rescuing lacZ-containing plasmids from genomic DNA. Detailed procedures for plasmid rescue have been described before (23). In brief, pUR288-lacZ reporter transgenic mice (line 60) (20). Immunodeficient mice were obtained by crossing WT, Xpa−/−, and Xpa+/− mice with DNA-PKcs knockout mice (21). To confirm genotypes, polymerase chain reaction analyses were performed as described previously (22). All mice were housed specific pathogen free in a climate-controlled room with a 12 h on/off light cycle. Feed and water were available ad libitum throughout the course of all experiments. The study was agreed upon by the institute’s Ethical Committee on Experimental Animals, in accordance to national legislation.

**Survival study with DNA-PKcs−/− mice**

DNA-PKcs−/−, DNA-PKcs+/−/Xpa−/− and DNA-PKcs+/−/Xpa−/−/p53−/− mice (further referred to as DNA-PKcs, DNA-PKcs/Xpa and DNA-PKcs/Xpa/p53, respectively) were monitored throughout their lifetime and body weight was measured weekly. When mice became moribund or when their body weight dropped by >20%, autopsy was performed. Spleen, MLNs, thymus, liver and tissues showing macroscopic abnormalities were isolated and subjected to pathologic and immunohistochemical analyses as described below.

**Characterization of lymphomas**

Spleen, MLNs and thymus of the CsA-exposed mice and the DNA-PKcs−/− mice were subjected to FACS analyses and immunohistochemistry. For FACS analyses, single-cell suspensions were prepared from the tissues and kept in RPMI medium. Cells were incubated for 10 min at room temperature with antibodies directed against CD3, CD4, CD8, B220 and CD45, which were diluted 1:100 in FACS buffer (1% bovine serum albumin and 0.1% NaN₃ in phosphate-buffered saline). Erythrocytes were lysed using FACS lysing solution and the remaining leucocytes were washed with phosphate-buffered saline supplemented with 2% fetal calf serum. Cells were fixed with 0.1% paraformaldehyde and kept at 4°C until FACS analysis. Cells were quantified using CellQuest software (Becton Dickinson, San Jose, CA). For immunohistochemistry, tissues were snap frozen in liquid nitrogen. Frozen sections of selected lymphomas were prepared for immunohistochemistry as described previously (24), using antibodies against CD3, CD4, CD19, CD45R and IgM. Samples of the spleen were used as a control for the immunohistochemical staining methods.

**Statistical analyses**

Statistical analyses were performed using SPSS 15.0 and S-Plus. The incidence of lymphomas in the CsA exposure studies was analyzed by performing a Fisher’s exact test. Differences in survivals of DNA-PKcs−/− mice were studied by Kaplan–Meier with log-rank test. To take into account the difference in survival rates, the incidence of lymphomas in DNA-PKcs−/− mice was analyzed using a Poly-3 test. Mutant frequencies were analyzed using a univariate model with treatment and genotype as fixed effects. For all tests, P-values were considered to be significantly different when P < 0.05.

**Results**

**Tumor incidence in Xpa/p53 mice following 39 weeks of CsA exposure**

WT, Xpa−/−, and Xpa+/− mice were fed CsA in their diet for 39 weeks in order to study carcinogenic features of the compound. In concordance with previous findings (5), CsA treatment resulted in a higher incidence of lymphomas in Xpa−/−/p53−/− mice compared with untreated mice or exposed WT mice (Table I). The CsA-induced lymphomas were of a pleomorphic type, resembling follicular center cell lymphoma (Figure 1A). As compared with follicular center cell lymphomas normally observed in mice, the CsA-induced lymphomas contained a relatively high number of cytoplasm-rich cells. We did not observe any differences in the CsA-induced tumor types between the three genotypes.

<p>| Table I. Lymphoma incidence in WT, Xpa and Xpa/p53 mice after 39 weeks exposure to CsA |
|---------------------------------|-----|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Dose (in p.p.m.)</th>
<th>WT</th>
<th>Xpa</th>
<th>Xpa/p53</th>
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<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>65</td>
<td>190</td>
</tr>
<tr>
<td>Males (n)</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Females (n)</td>
<td>15</td>
<td>14</td>
<td>15</td>
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</table>

*Column represents results of second short-term carcinogenicity study.
*Significantly increased as compared with corresponding controls, P < 0.05.
*Significantly increased as compared with corresponding controls, P < 0.01.
*Significantly increased as compared with both CsA-treated WT (P < 0.001) and Xpa (P < 0.01) mice.
*Significantly increased as compared with CsA-treated WT mice, P < 0.05.
*Significantly increased as compared with Xpa mice, P < 0.05.
Other tumor types, besides lymphoma, found in CsA-treated mice included bronchioloalveolar adenoma, osteosarcoma, hemangiosarcoma of the spleen and liver, adenocarcinoma or adenomatous polyp in small intestine. These tumors were found sporadically and were found equally distributed over the genotype groups. They also belong to the spontaneous tumor background of C57BL/6 mice (5) and are probably not treatment related.

CsA is not genotoxic to DNA repair-deficient Xpa/p53 mice
To prove that CsA is non-genotoxic, even in DNA repair-deficient mice, lacZ mutant analyses were performed on spleen, MLNs and liver of Xpa and Xpa/p53 mice. No significantly increased lacZ mutant frequencies were found in any of the tissues analyzed, confirming that CsA is a non-genotoxic carcinogen, even in DNA repair-deficient mice (Figure 2). 2-Acetylaminofluorene control treatment resulted in a mutant frequency of $6.5 \times 10^{-5}$ in the liver (data not shown), a significant increase as compared with untreated mice ($P < 0.01$). These results are clearly in line with our previous findings (25).

Tumor incidence in DNA-PKcs/Xpa/p53 mice
To investigate the effect of immunosuppression through a genetic approach, mice were crossed with DNA-PKcs-deficient mice. Lymphoma incidence in DNA-PKcs-deficient WT, Xpa and Xpa/p53 mice was monitored. Survival, tumor incidence and presence of T and B cells in spleen, MLNs and thymus were studied in all genotypes. As shown in Figure 3, DNA-PKcs/Xpa/p53 mice have a shorter life span as compared with the other genotypes ($P < 0.001$). In addition, the incidence of lymphomas was significantly increased in this genotype, taking differences in life span into account (Table III, $P < 0.001$). In all genotypes, lymphomas were found, in which females appear more sensitive in WT and Xpa mice. Histologically, the lymphomas found in DNA-PKcs-deficient mice differed from the CsA-induced lymphomas. They were generally of the lymphoblastic type, showing a uniform picture of relatively large cells with large nuclei (Figure 1B). The distribution of T and B cells in spleen, MLNs and thymus invaded by lymphoma was analyzed by FACS analyses and immunohistochemical staining. The distribution of T and B cells in the lymphomas of DNA-PKcs/Xpa/p53 mice was similar to the distribution found in lymphomas of CsA-treated Xpa/p53 mice, specifically in the spleen (Table II, compare block 1 and 2). Spleens invaded with lymphoma consisted mainly of B cells, whereas one lymphoma in the thymus consisted clearly of T cells. Immunohistochemistry on a representative number of lymphomas confirmed these results.

Interestingly, another tumor type besides lymphoma was found to be genotype specific: six of 15 DNA-PKcs/Xpa/p53 females had developed an adenocarcinoma in the mammary gland (Figure 1C, Table III). No tumors in the mammary gland were found in the other genotypes.

Other tumors found were hepatocellular adenoma, histiocytic sarcoma in the liver and lymphoid system and bronchioloalveolar adenoma. These were found in all genotypes and are probably not related to the immune-deficient status of the mice.

Discussion
Xpa/p53 mice were developed to be used as an alternative to the chronic rodent bioassay. Short-term carcinogenicity studies revealed that this model has an increased sensitivity to develop cancer after exposure to genotoxic but unexpectedly also to human non-genotoxic carcinogens (5). In the study presented here, we investigated the underlying mechanism of this remarkable feature, using CsA as the challenging agent.

Our analyses revealed that CsA is a non-genotoxic compound, even in DNA repair-deficient mice. However, we cannot rule out the possibility that CsA induces loss of heterozygosity of the WT p53 allele in the ultimate tumor. These kinds of mutations are hardly detectable in pUR288 LacZ reporter mice. Subsequently, we focused on the incidence and the phenotype of tumors induced by CsA. The increased incidence of lymphomas in Xpa/p53 mice upon exposure to CsA,
**Table II.** FACS and immunohistochemical analyses of spleen (Sp), MLN and thymus (Th)

<table>
<thead>
<tr>
<th></th>
<th>Xpa/p53, CsA treated</th>
<th>DNA-PKcs/Xpa</th>
<th>DNA-PKcs/Xpa</th>
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<tr>
<td></td>
<td>Sp</td>
<td>MLN</td>
<td>Th</td>
<td>Sp</td>
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<tr>
<td>Number lymphomas</td>
<td>10</td>
<td>5</td>
<td>1</td>
<td>9</td>
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<td>Mainly T cells</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Mainly B cells</td>
<td>7</td>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B and T cells equal</td>
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<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Inconclusive/no data</td>
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<td>1</td>
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</tr>
</tbody>
</table>

**Fig. 2.** *LacZ* mutant frequencies in (A) liver, (B) spleen and (C) MLNs of untreated and CsA-treated WT, *Xpa* and *Xpa/p53* mice. Mice were fed 500 p.p.m. of CsA for 12 weeks or were untreated for 6 weeks. Mouse treatment with 300 p.p.m. of 2-acetylaminofluorene, used as a control, resulted in a mutant frequency of $65 \times 10^{-5}$ in the liver (data not shown). Error bars indicate standard deviation of the mean.

observed in a previous study (5), was confirmed. As was shown previously, lymphoma incidence in *Xpa* mice was increased in the highest CsA dose group. In a 6 months exposure study with CsA in *p53* 

with previous findings, where CsA was reported to induce lymphomas in both humans and rodents (5,12,13). CsA-induced lymphomas developed mostly in the spleen and consisted of mainly B cells. Normal spleens not invaded by lymphoma had equal numbers of T and B cells, demonstrating that there is B cell enrichment in CsA-induced lymphomas.

In subsequent experiments, we concentrated on the immunosuppressive effect of CsA and studied the effect of immunosuppression itself on lymphoma incidence in *Xpa/p53* mice. For this, we used a genetic approach by crossing WT, *Xpa* and *Xpa/p53* mice with DNA-PKcs-deficient mice. These DNA-PKcs-deficient mice are unable to perform proper V(D)J recombination, thereby preventing development of mature T and B cells (21,27,28). DNA-PKcs/*Xpa/p53* mice have a shorter life span as compared with the other genotypes. Since *p53* 

DNA-PKcs-deficient mice have no mature T and B cells at all. In CsA-treated mice, the DNA-PKcs/*Xpa/p53* mice developed significantly more lymphomas as compared with the other two genotypes. Moreover, T and B cell distribution in lymphomas was comparable with the situation as observed in CsA-induced lymphomas. In both studies, the dominant cell type in the spleen was the B cell, whereas in the thymus mostly T cells were found. Despite the fact that DNA-PKcs-deficient mice lack mature B and T cells, we detected cells positive for B and T cell markers in the lymphomas. The B cell-positive result can be explained by the presence of pre-B cells instead of mature B cells. Indeed, the B cell tumors we found were negative for IgM, which is only present on mature B cells.

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Lymphomas are frequently found in DNA-PKcs-deficient mice and Scid mice and are of a T cell phenotype residing in the thymus (32,33). In our DNA-PKcs/*Xpa/p53* mice, the majority of lymphomas was found in the spleen and consisted mostly of B cells rather than T cells. Previous studies with *p53*-deficient mice also showed a majority of B cell lymphomas, indicating that the shift toward B cells in *Xpa/p53* mice might be due to the *p53* haploinsufficiency (34,35). The above-mentioned studies (32,33) leading to T cell lymphomas were done in a *p53* WT background; therefore, we believe that splenic B cell lymphomas originated as a consequence of *p53* haploinsufficiency. This does not necessarily imply, however, that these tumors arose due to hampered immunosurveillance.

Although a comparable distribution of T and B cells was found in lymphomas of DNA-PKcs-deficient mice and CsA-treated mice, lymphomas in DNA-PKcs-deficient mice were somewhat different in morphology. In DNA-PKcs-deficient mice, the lymphomas were more uniform and consisted mainly of lymphoblastic cells. The tumor phenotype of CsA-treated mice was more pleomorphic and lymphomas consisted mainly of follicular center cells. This difference is not surprising as immunodeficiency can be initiated in several ways. DNA-PKcs mice have no mature T and B cells at all. In CsA-treated mice, which do have mature T and B cells, lymphoid cell proliferation is...
development is blocked, leading to a dysfunctional immune system. It
in the CsA-treated and DNA-PKcs-deficient mice, T and B cell
munosurveillance is still intact in these mice. The immune system can
tumor background (4). This is most probably due to the fact that im-
found in the mammary gland of CsA-treated mice. We speculate that
immunosurveillance. For unknown reasons, these tumors were not
increased lymphoma incidence in both CsA-treated mice and DNA-
PKcs-deficient mice is mainly found in the Xpa/p53; mice. These
Xpa; mice. These initiated cells are normally neutralized through
exclusively found in DNA-PKcs/ mice.

### Table III. Tumor incidence in DNA-PKcs, DNA-PKcs/Xpa and DNA-PKcs/
Xpa/p53 mice

<table>
<thead>
<tr>
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<tr>
<td>Mice examined, males (n)</td>
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<td>13</td>
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<tr>
<td>Lymphomas (n)</td>
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<td>0</td>
<td>5*</td>
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<tr>
<td>Mice examined, females (n)</td>
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<tr>
<td>Mammary adenocarcinomas (n)</td>
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<td>5</td>
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<tr>
<td>Mammary adenocarcinomas (n)</td>
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<td>0</td>
<td>6*</td>
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<tr>
<td>Mice examined, total (n)</td>
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<td>23</td>
<td>28</td>
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<tr>
<td>Lymphomas (n)</td>
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<td>10*</td>
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<tr>
<td>Mammary adenocarcinomas (n)</td>
<td>0</td>
<td>0</td>
<td>6*</td>
</tr>
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</table>

*Significantly increased as compared with DNA-PKcs and DNA-PKcs/Xpa mouse (Fisher’s exact test, P < 0.01 and P < 0.05, respectively).

*Significantly increased as compared with DNA-PKcs mice (Poly-3 test, P < 0.001).

### References


### Table III

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>DNA-PKcs</th>
<th>DNA-PKcs/Xpa</th>
<th>DNA-PKcs/Xpa/p53</th>
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<tr>
<td>Mice examined, males (n)</td>
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<td>13</td>
<td>13</td>
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<tr>
<td>Lymphomas (n)</td>
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<td>Mammary adenocarcinomas (n)</td>
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<td>5</td>
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<td>Mammary adenocarcinomas (n)</td>
<td>0</td>
<td>0</td>
<td>6*</td>
</tr>
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</table>

*Significantly increased as compared with DNA-PKcs and DNA-PKcs/Xpa mouse (Fisher’s exact test, P < 0.01 and P < 0.05, respectively).

*Significantly increased as compared with DNA-PKcs mice (Poly-3 test, P < 0.001).


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