TLR9-agonists oppositely modulate DNA-repair genes in tumor versus immune cells and enhance chemotherapy effects

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ABSTRACT

Synthetic oligodeoxynucleotides expressing CpG motifs (CpG-ODN) are a TLR9 agonist that can enhance the anti-tumor activity of DNA-damaging chemotherapy and radiation therapy in preclinical mouse models. We hypothesized that the success of these combinations is related to the ability of CpG-ODN to modulate genes involved in DNA-repair. We conducted an in silico analysis of genes implicated in DNA-repair in datasets obtained from murine colon carcinoma cells in mice injected intratumorally with CpG-ODN and from splenocytes in mice treated intraperitoneally with CpG-ODN. CpG-ODN treatment caused down-regulation of DNA-repair genes in tumors. Microarray analyses of human IGROV-1 ovarian carcinoma xenografts in mice treated i.p. with CpG-ODN confirmed in silico findings. When combined with the DNA-damaging drug cisplatin, CpG-ODN significantly increased the lifespan of mice compared to individual treatments. In contrast, CpG-ODN led to an up-regulation of genes involved in DNA-repair in immune cells. Cisplatin-treated ovarian carcinoma patients as well as anthracycline-treated breast cancer patients that are classified as "CpG-like" for the level of expression of CpG-ODN modulated DNA-repair genes have a better outcome when compared to patients classified as "CpG-untreated-like", indicating the relevance of these genes in the tumor cell response to DNA-damaging drugs. Taken together, the findings provide evidence that the tumor microenvironment can sensitize cancer cells to DNA-damaging chemotherapy, thereby expanding the benefits of CpG-ODN therapy beyond induction of a strong immune response.
INTRODUCTION

The mammalian innate immune system identifies the presence of infection through recognition of pathogen-associated molecular patterns (PAMPs) expressed by a diverse group of infectious microorganisms. Various pattern recognition receptors are involved in PAMP identification, among which the toll-like receptor (TLR) family of at least 10 different members in humans (1;2) is probably the best known. These receptors are considered sensors for microbial infections or other “danger signals” and, together with other molecular sensors, serve as a first line of defense, inducing soluble and cellular mediators of innate immunity and initiating key steps of the adaptive immune response (3). Particular clinical interest now revolves around TLR9, which is expressed not only on cells of the immune system but also on endothelial cells, fibroblasts, and epithelial cells (3-6) and which recognizes bacterial and viral DNA with unmethylated CpG motifs. Synthetic oligodeoxynucleotides (ODN) expressing CpG motifs mimic the immune-stimulatory activity of bacterial DNA and are commonly used to activate TLR9 for therapeutic applications (3). CpG-ODN have demonstrated anti-tumor activity in different animal models (7;8) and in patients with malignant melanoma, renal carcinoma and recurrent or refractory lymphoma (9-12); however, experimental studies suggest that CpG-ODN may be more useful as a component of multi-agent therapy for cancer rather than as a single agent (13). Since chemotherapy is known to be immunosuppressive, it may seem counterintuitive to combine it with TLR9 stimulation. Nevertheless, different chemotherapeutic drugs have been reported to improve the efficacy of CpG-ODN in mouse tumor models (14;15). In immunocompetent mice, paclitaxel and
cyclophosphamide enhanced CpG-ODN effects by depleting regulatory T cells, increasing the immunogenicity of tumor cells, and changing T cell homeostasis; moreover, the presence of CD8⁺ T cells was found to be required (16). In immunocompromised athymic mice, the mechanisms underlying the improved anti-tumor effect when CpG-ODN was combined with gimatecan (17), gemcitabine (6), or topotecan (18;19) have to be different from those in immunocompetent mice and remain unclear. It is noteworthy that, although these three chemotherapeutic agents differ in mechanisms of action, their cytotoxic activity is generally the consequence of DNA damage. Even ionizing radiotherapy (RT), generally administered locally to the tumor site or draining lymph nodes, kills cancer by damaging DNA, and experiments in murine models suggest that CpG-ODN can enhance the response to RT in both immunogenic (20;21) and non-immunogenic tumors (22). For non-immunogenic tumors, the mechanism remains undefined since the rationale for even exploring the combination of CpG-ODN with radiation, i.e., that dendritic cells (DC) acquire antigens released from tumor cells after RT and migrate to regional lymph nodes where they encounter and activate tumor-specific cytotoxic cells, does not apply.

Unlike observations in tumor cells where CpG-ODN appears to increase the activity of RT, studies on the combination of RT and CpG-ODN in mice have shown that TLR9 engagement on CD4⁺ T lymphocytes reduces apoptosis and enhances their capacity to repair DNA damage induced by gamma-radiation (23).

Based on these disparate observations, we hypothesized that CpG-ODN modulate genes involved in DNA repair, increasing their expression in TLR9-expressing immune cells, but down-regulating their expression in tumor cells and thereby increasing sensitivity to
DNA-damaging chemotherapeutic agents. To test this hypothesis, we analyzed the effect of CpG-ODN treatment on DNA repair gene expression in immune and tumor cells *in silico* and in our previously described model of IGROV-1 ovarian tumor-bearing athymic mice (24), and assessed the anti-tumor effect of CpG-ODN associated with DNA-damaging chemotherapy in this mouse model.
MATERIALS AND METHODS

Cell lines
The IGROV-1 tumor (gift from Dr. J. Benard, Institute Gustave Roussy, Villejuif, France) and OVCAR-5 (American Type Culture Collection) were adapted to grow i.p. and maintained by serial i.p. passage of ascitic cells into healthy mice as previously described (25). Every six months, cells were authenticated by morphologic inspection and by the presence of specific markers with FACS analysis. For in vitro experiments, cells were maintained in RPMI medium 1640 supplemented with 10% FCS (Sigma) and 2 mM glutamine (Cambrex, East Rutherford, NJ) at 37°C in a 5% CO₂ air atmosphere.

In vitro and in vivo experiments for microarray analyses
For microarray experiments, mice were injected i.p. with 2.5 x 10⁶ ascitic cells in 0.2 ml of saline and treated starting 11-12 days later, when mice showed evident and established ascites, with CpG-ODN delivered i.p. at a dose of 20 μg/mouse daily for 3 days. Control mice received saline. Ascites-bearing mice were sacrificed by cervical dislocation at 24 h hours after the last treatment with saline or CpG-ODN. Tumors adherent to omentum were removed and immediately frozen in liquid nitrogen until RNA or protein extraction. For microarray experiments, to evaluate a direct action of CpG-ODN on tumor cells, 1x10⁶ IGROV-1 cells were seeded in 6-well plates and treated with 10 μM of CpG-ODN in complete culture medium for 24 h. At the end of treatment, cells were collected and RNA extracted.
To define whether local treatment at the tumor site is critical to down-regulate DNA repair genes, mice were injected i.p. with IGROV-1 tumor cells as described above and treated i.p. or subcutaneously (s.c.) with CpG-ODN at a dose of 20 μg/mouse daily for 3 days. At 24 h after the last treatment with saline or CpG-ODN, ascites-bearing mice were sacrificed and tumors adherent to the peritoneal wall were removed and immediately frozen in liquid nitrogen until RNA extraction.
RESULTS

DNA repair gene modulation in immune and non-immune normal cells by CpG-ODN

To evaluate the effect of CpG-ODN on DNA repair genes in immune cells, we conducted a comprehensive in silico expression analysis of genes implicated in DNA repair (GSE11202) in immune spleen cells from mice treated i.p. with CpG-ODN (26). Spleen cells were chosen for this analysis based on previous studies establishing that the spleen accurately reflects the breadth of immunity induced by CpG-ODN in vivo (27-32). mRNA expression levels in mouse spleen cells were monitored by microarray at different times after in vivo CpG-ODN treatment. From a list of 209 genes retrieved according to the “DNA repair” term from Gene Ontology (GO:0006281 mouse), 189 were present in the GSE11202 and 49 genes were found to be significantly modulated (FDR<0.05) during the course of CpG-ODN treatment, 43 of which were up-regulated (Figure 1). Accordingly, analysis of a published gene expression dataset (E-TABM-823) in immune mucosal lung tissue of mice 48 h after intranasal administration of CpG-ODN, when CpG-ODN induced recruitment of natural killer (NK) and DC into the bronchoalveolar spaces peaked (33), identified 29 genes in the DNA repair pathway that were significantly modulated by CpG-ODN treatment (FDR<0.05), 21 of which were up-regulated (Supplementary Figure 1). Noteworthy, 13 of the 29 modulated genes were shared with those modulated in immune spleen cells. Thus, the presence of microbial DNA appears to induce up-regulation of genes involved in DNA repair in immune cells.

To test whether CpG-ODN similarly affects non-immune TLR9-negative normal cells, we conducted expression analysis of DNA repair genes using a whole-mouse genome microarray dataset (E-TABM-506) obtained from murine quadriceps muscle injected
with CpG-ODN in experiments to evaluate this bio-drug as a vaccine adjuvant (34); only 2 genes, NBR and FAM175, for which the specific function of the encoded proteins is unknown, were modulated (FDR <0.05) (data not shown). Thus, CpG-ODN does not induce significant modulation of DNA repair genes in non-immune normal cells.

**DNA repair gene modulation in tumor cells by CpG-ODN**

The potential effect of CpG-ODN in tumor cells was evaluated based on the expression of the corresponding GO:0006281mouse genes in a dataset obtained from MC38 murine colon carcinoma cells in mice injected intratumorally with CpG-ODN (35) (GSE18203). CpG-ODN treatment was found to modulate DNA repair gene expression in tumors and 50 genes were modulated at a threshold of p<0.05. However, unlike observations in immune cells, CpG-ODN treatment induced mainly a down-regulation of DNA repair genes in tumor cells (40 of 50 modulated genes were down-regulated) (Figure 2 A and B). These findings were experimentally proofed by microarray analyses of human IGROV-1 ovarian carcinoma xenografts in mice treated daily i.p. with CpG-ODN or saline beginning at 3 days after evidence of ascites. At 24 h after the final treatment, tumors adhering to omentum were collected, and RNA extracted from the tumors cells was analyzed for gene expression profile. Among the 232 genes belonging to GO:0006281human, 227 genes available in our microarray platform clustered tumors according to saline or CpG-ODN treatment (Figure 3A) (GSE23441), and the pattern of this gene modulation in CpG-ODN-treated mice reflected an increased susceptibility to DNA damage (75 of 114 genes modulated at a threshold of p<0.05, were down-regulated) (Figure 3B). The power of CpG-ODN in modulating cancer cell DNA repair genes was confirmed by Ingenuity Pathway Analysis (IPA), since IPA of 1765 up- or
down-modulated genes by CpG-ODN compared to controls revealed that the “Cell Death, Embryonic Development, DNA Replication, Recombination, and Repair pathway” was the most influenced (Supplementary Figure 2). qRT-PCR of RAD51C, SIRT1, RAD54B and RAD23B genes validated microarray data (Supplementary Figure 3).

Results of microarray analyses were also validated by examining on tumors from CpG-ODN-treated and control mice the expression of the gene products RAD51 and SIRT1. Western blotting analysis of tumor cell lysates indicated decreased expression of RAD51, and SIRT1 proteins in treated mice as compared to controls (Figure 4 A and B). Thus, microarray analyses indicate that locally administered TLR9 agonists regulate genes involved in DNA repair in tumor cells in the opposite way than in immune cells.

Expression of CpG-ODN modulated DNA repair genes and survival in ovarian and breast cancer patients treated with DNA damaging therapy

To evaluate whether CpG-ODN induced DNA repair gene modulations, observed in IGROV1 microarray analysis, were relevant to increase the cell sensitivity to DNA-damages, among the genes found differentially modulated between the CpG-ODN treated and untreated IGROV-1 tumors, a set of 27 genes with a level of FDR<0.01 and a fold difference >1.5 was selected (Supplementary Figure 4) and the average expression of both CpG-ODN treated and untreated tumors was calculated for each gene; the resulting expression pattern was used to analyze an ovarian microarray dataset containing the gene expression data of ovarian cancer samples obtained at initial cytoreductive surgery from patients who then received platinum-based primary chemotherapy (36). The Pearson
correlation coefficient between expression values of the 27 genes and those available in the ovarian dataset was calculated for both the CpG-ODN-treated and untreated conditions; the difference was tested for its association with clinical outcome in Kaplan-Meier survival analysis, with patients grouped depending on correlation values. Two groups were identified as CpG-ODN-treated-like cases (named “CpG-like”) and CpG-ODN-untreated like cases (named “CpG-untreated-like”). Analysis of dataset for available clinical data revealed no association of “CpG-like” feature with grade and debulking (Supplementary Table 1A).

As shown in Figure 5A, patients of the “CpG-like” group showed a significantly increased overall survival compared to the “CpG-untreated-like” group. Cox’s proportional hazard analysis confirmed above mentioned results (HR=0.5949, 95%CI=0.3663-0.9661, p-value=0.0367). Multivariate Cox’s proportional model indicated that “CpG-like” signature resulted independently associated with overall survival (HR=0.5749, 95%CI=0.346-0.9553, p=0.0335) (Supplementary Table 1B). A cross-validation procedure removing each single gene from the gene set and performing the Cox-analysis on the remaining gene set revealed that the prediction performance is not related to a particular gene, since in all analyses a p-value<0.05 was observed (Supplementary Figure 5) even though RAD23B is the gene that mainly influences the significance of statistical analysis.

To test the relevance of RAD23B in the sensitivity to DNA-damaging agents in IGROV-1 cell line, we down-regulated RAD23B protein levels by specific siRNA transfection (Supplementary Figure 6A). In vitro cisplatin cytotoxicity is significantly reduced (p<0.0001) by down-regulation of RAD23B (Supplementary Figure 6B).
When Kaplan-Meier survival analysis was performed on a breast cancer dataset of whole-genome expression of patients who received adjuvant DNA-damaging chemotherapy after surgery (37), patients classified as “CpG-like” showed a significantly increased relapse-free and overall survival compared to “CpG-untreated-like” patients (Figure 5B); on the contrary, no outcome difference was observed in the cohort of patients not treated with DNA damaging adjuvant therapy (Figure 5C).

In chemotherapy treated patients, “CpG-like” signature was significantly associated with grade (1+2) (p=0.0048), whereas no association was found with the other pathologic parameters (estrogen receptor, tumor size and node status) (Supplementary Table 2A). Multivariate analysis of all covariates (i.e. estrogen receptor, tumor size, grade, node status and “CpG-like” signature) indicated that CpG-like signature resulted an independent strong prognostic factor (HR=0.3875, 95%CI=0.1812-0.8287, p=0.015) of relapse-free survival (Supplementary Table 2B).

Altogether, these findings indicate that CpG-ODN modulate DNA repair gene expression that are relevant for cell sensitivity to DNA-damages.

**Antitumor effect of CpG-ODN and cisplatin in human ovarian tumor xenografts**

To test for a correlation between DNA repair gene down-modulation and sensitivity to DNA damage-inducing drugs, we evaluated the effect of CpG-ODN treatment on the anti-tumor activity of cisplatin, a DNA cross-linking agent (38). IGROV-1 ovarian tumor-bearing athymic mice were used in these experiments, since IGROV-1 cells are sensitive to cisplatin (39) and since CpG-ODN in this model has been shown to prolong survival of mice with bulky disease inducing an activation of different effector cells and
cytokines of innate immunity at the site of tumor growth (8;24). Mice were treated i.p. with CpG-ODN, cisplatin, or both at 8 days after tumor cell injection, when ascites starts to form. Analysis of the effect of the combined treatment revealed a significant (p<0.0001) increase in lifespan compared to the use of either reagent alone (T/C values: 200% for cisplatin, 305% for CpG-ODN, 460% for cisplatin plus CpG-ODN) (Figure 6).

The antitumor efficacy of CpG-ODN in combination with cisplatin was also assayed in mice bearing the human ovarian tumor cells OVCAR-5. As previously observed in IGROV-1 mouse model, mice bearing OVCAR-5 human ovarian cell line and treated with CpG-ODN plus cisplatin survive significantly longer than those treated with CpG-ODN or cisplatin alone (T/C values: 118% for cisplatin, 188% for CpG-ODN, 267% for cisplatin plus CpG-ODN) (Supplementary Figure 7).

It should be noted that the modulation of DNA repair genes in human ovarian IGROV-1 tumors and the increase in the anti-tumor effect of cisplatin and CpG-ODN against IGROV-1 human ovarian tumor xenografts in mice were observed in mice injected with a CpG-ODN sequence specific for murine TLR9, making unlikely the possibility that the modulation was related to a direct interaction of CpG-ODN with tumor cells since different DNA motifs are required for stimulation of mouse and human cells by CpG-ODN.(40;41). Nevertheless, we carried out a microarray analysis on mRNA extracted from IGROV-1 cells stimulated in vitro with murine CpG-ODN (GSE23442); none of 16824 analyzed genes was found to be significantly modulated by CpG-ODN (no genes with FDR<0.1) compared with IGROV-1 cells cultured in medium alone (data not shown), excluding a direct action of CpG-ODN on tumor cells.
Our evidence of down-modulation in DNA repair genes in tumor cells in the analyses thus far involved the administration of CpG-ODN at or near the tumor site. Indeed, microarray analyses of mRNA from IGROV-1 tumors adhering to the omentum in mice bearing IGROV-1 ascites and treated subcutaneously daily for 3 days with CpG-ODN revealed no significantly modulated genes (with FDR<0.1) as compared to tumors from saline-treated mice (data not shown); thus, injection of CpG-ODN at the tumor site appears to be critical for DNA repair gene modulation in tumor cells.

In that case, a relevant role for peritumoral TLR9-expressing cells, such as innate immune cells, and/or endothelial cells, fibroblasts and epithelial cells, in this modulation is plausible. These cells might induce down-regulation of DNA repair genes in tumor cells through a direct cell-cell interaction and/or by secreting soluble factors.

To evaluate the involvement of soluble molecule(s) in DNA repair gene-modulation, cell-free ascitic fluid supernatants, obtained from mice treated i.p. daily for 3 days with CpG-ODN or saline were added to cultured IGROV-1 cells. Forty-eight h later mRNA was extracted and the mRNA levels of RAD23B, RAD51C, SIRT1 and RAD54B were determined by qRT-PCR. Incubation with ascitic fluids from CpG-ODN treated mice induced a down modulation of RAD51C (p=0.0443), SIRT1 (p=0.0374) and RAD54B (p=0.00851) levels, consistent with microarray results, and a trend toward a higher median expression level for RAD23B (Supplementary Figure 8), which was up-modulated in microarray experiments.

Moreover, pre-incubation of IGROV-1 cells with ascitic fluids from CpG-ODN-treated mice for 48h enhanced cisplatin cytotoxicity, since cell viability quantified by MTT assay was 66.0±4.6% (mean±SEM) for cells pre-incubated with ascitic fluid from CpG-ODN-
treated mice and 90.1±5.8% for cells pre-incubated with ascitic fluids from saline treated mice (p=0.0121).

**DNA repair gene modulation in tumors by TLR7-agonist imiquimod**

Furthermore, to determine whether modulation of DNA repair genes in tumor cells is restricted to CpG-ODN induced TLR9 activation or occurs after activation with the only other TLR agonist currently used in oncological therapy, the imiquimod, an immunomodulator that functions as agonist of TLR7 and/or TLR8 (42), a comprehensive *in silico* expression analysis of genes implicated in DNA repair (GO:0006281mouse) on mRNA extracted from spontaneous s.c. tumors from mice treated topically with imiquimod (43) (GSE20032) was performed. Imiquimod treatment was found to modulate DNA repair gene expression in tumors, which clustered according to treatment (treated vs. control) (Supplementary Figure 9A), and the pattern of gene modulation in imiquimod-treated mice reflected an increased susceptibility to DNA damage (29 of 32 genes modulated at a threshold of p<0.05 were down-regulated) (Supplementary Figure 9B).
DISCUSSION

In the present study, we show by microarray analyses that TLR9-ligand CpG-ODN treatment induces down-modulation of DNA repair genes in tumor cells of both murine and human origin. Expression level analysis of proteins, RAD51 a key protein in the homologous recombination DNA repair pathway (44) and SIRT1 whose activity promotes homologous recombination (45), in human tumor cells confirmed microarray results. These proteins are involved in homologous recombination and, consequently, are relevant for the repair of interstrand cross-links, which are the most cytotoxic lesions induced by cisplatin. Accordingly, the combination of cisplatin and CpG-ODN against IGROV-1 human ovarian tumor xenografts in athymic mice was found to induce a remarkable increase in lifespan compared to that using either reagent alone (p<0.0001).

The combined data from our and *in silico* analyses revealed modulation of DNA repair genes in both murine colon carcinoma MC38 cells and human ovarian carcinoma IGROV-1 cells, suggesting that this effect is not restricted to a specific cancer cell histotype. The observation that this modulation also occurs after treatment with an agonist of TLR7 and/or TLR8, might suggest that this event can be shared by the functional subgroup of TLRs, consisting of TLR7, 8 and 9, which reside intracellularly and recognize nucleic acids derived from the genome of viruses and bacteria (42).

The findings that intratumoral delivery of CpG-ODN was critical in inducing DNA repair protein down-modulation in tumors and that CpG-ODN did not interact directly with the tumor cells to induce this down-modulations in IGROV-1 ovarian cancer cells point to the importance of the activation of the TLR9 positive cells (innate immune cells,
endothelial cells, fibroblasts, and epithelial cells) present in the tumor microenvironment, in modulating DNA repair gene expression. Moreover, our data suggest that soluble molecules in ascitic fluid, presumably released by the above-mentioned cells TLR9-positive cells, are involved in the modulation of DNA-repair genes. The lower modulation of DNA repair genes observed in ex vivo experiments compared with that in in vivo might depend on the loss of activity of some factors or on the need for both soluble factors and contact between CpG-ODN-activated cells and tumor cells.

Clinical results from association of CpG-ODN with DNA-damaging drugs in lung tumor patients have so far been disappointing in improving clinical outcome (46).

Based on our findings, this absence of CpG-induced enhanced chemosensitivity of tumor cells to DNA damage-inducing agents may rest in the subcutaneous administration of CpG-ODN, distant from the tumor cells.

On the contrary, we observed by in silico analyses that TLR9-ligand CpG-ODN treatment induces essentially an up-modulation of DNA repair genes in immune cells. Moreover, it is noteworthy that 19 of 49 genes modulated in spleen cells after CpG-ODN treatment, resulted modulated even in IGROV-1 tumor cells, but for most of them the modulation was in an opposite way (14 up-regulated in spleen were down-regulated in tumor and 3 down-regulated in spleen were up-regulated in tumor).

Despite numerous previous microarray analyses of immune cells after in vitro or in vivo stimulation with CpG-ODN (26;33;35;43;47), to our knowledge an up-regulation of DNA repair genes after CpG-ODN treatment has not been reported. This might reflect a primary focus in previous studies on signaling pathways that induce expression of
immune and pro-inflammatory genes (48), in light of the function of TLRs as innate immunity sensors of microbial products.

CpG-ODN-induced down-modulation of DNA repair genes in tumor cells and up modulation in immune cells might represent a physiological phenomenon that occurs locally in the presence of an infectious event. Thus, upon detecting the presence of an infectious agent via endosomal TLRs, immune cells might regulate DNA repair genes to decrease their susceptibility to possible pro-apoptotic signals during infections and, at the same time, directly and/or indirectly induce modulation of DNA repair genes in infected (or transformed) cells to facilitate their death. Insignificant modulations of these genes observed in normal muscle cells suggest that CpG-ODN activated immune cells induce down-modulation of DNA repair genes only in “altered” cells expressing apposite receptors. Moreover, better outcome of cisplatin-treated ovarian carcinoma patients, as well as of anthracycline-treated breast cancer patients, classified as “CpG-like”, compared to patients classified as “CpG-untreated-like”, indicates the relevance of these genes in the tumor cell response to DNA-damaging drugs. In subjects who not received adjuvant chemotherapy, liker expression of CpG-ODN modulated DNA repair genes was not associated with significantly better outcome, indicating that these genes are not prognostic of survival.

Together, our present data provide the first evidence that TLR9-expressing cells present in the tumor microenvironment can sensitize cancer cells to DNA-damaging chemotherapy, underscoring the need for further investigation of the synergistic effect of CpG-ODN in combination with DNA-damaging drugs in cancer treatment.
ACKNOWLEDGMENTS

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REFERENCES


FIGURE LEGENDS

Figure 1. Microarray analysis of DNA repair pathway genes in spleen cells from CpG-ODN-treated mice. Mice were treated i.p. with CpG-ODN. RNA was extracted from spleen cells 1, 3, 9, 24 and 72 h after treatment and analyzed in-house-assembled oligonucleotide microarray platform. Of 209 genes involved in the DNA repair pathway (GO:0006281mouse), 189 were present in the GSE11202 dataset and 49 of these genes showed significant modulation (FDR<0.05) compared to that in untreated control mice (0 h). Color coding for each gene is normalized to the mean of the arrays for untreated controls (0 h). Black represents no change compared to controls; green and red represent down- and up-regulation with respect to the first time point, respectively. Changes from green to red to green indicate initial downregulation, increased expression, and final downregulation, respectively. Missing values are in yellow. Each row represents a sample and each column, a gene.

Figure 2. Microarray analysis of DNA repair pathway genes in MC38 murine colon tumors from CpG-ODN-treated mice. Mice bearing subcutaneous MC38 tumors were injected intratumorally with CpG-ODN or saline (control group). RNA extracted from tumors 6 h after treatment was analyzed on Affimetrix Platform using Affimetrix Mouse gene 1.0 ST chips; 201 genes in the DNA repair pathway (GO:0006281mouse) were detected in the Affimetrix Mouse Array (GSE18203). (A) Unsupervised hierarchical clustering of tumors according to the expression levels of 201 DNA repair genes. (B) Heat-map of modulated genes, 40 down- and 10 up-modulated (threshold p<0.05), in
CpG-ODN-treated mice (red: up-regulated genes; green: down-modulated genes). Each row represents a sample and each column, a gene.

**Figure 3. Microarray analysis of DNA repair pathway genes in IGROV-1 ovarian tumors from CpG-ODN-treated athymic mice.** IGROV-1-bearing mice with established ascites, i.e., increased abdominal volume and body weight, were treated i.p. daily for 3 days with CpG-ODN or saline (control group) and sacrificed 24 h later. RNA, extracted from tumors was analyzed on Illumina human whole-genome beads chips; 227 genes in the DNA repair pathway (GO:0006281human) were detected in our microarray experiment. (A) Unsupervised hierarchical clustering of tumors according to expression levels of 227 DNA repair genes. (B) Heat-map of modulated genes, 75 down- and 39 up-modulated (threshold p<0.05), in CpG-ODN-treated mice; (red: up-regulated genes; green: down-modulated genes). Each column represents a sample and each row, a gene.

**Figure 4. Western blot analysis of DNA repair proteins in IGROV-1 tumor cells adhering to the peritoneal wall after i.p. injection of CpG-ODN.** Protein expression level of SIRT-1 (A), Rad51 (B) in IGROV-1 ovarian cancer cells from athymic mice treated daily for 3 days with CpG-ODN or saline (4 mice/group). Vinculin was used to normalize protein loading per lane.

**Figure 5. Association between DNA-repair genes modulated by CpG-ODN and survival in patients treated with chemotherapy in an adjuvant setting.** Two datasets on whole-genome gene expression profiling of ovarian and breast cancer patients treated
with chemotherapy were chosen. A Pearson correlation coefficient was calculated to assess the correlation between each tumor and the CpG-ODN signature. The correlation was determined based on expression values of the 27 genes found modulated, comparing the CpG-ODN treatment versus the control condition, and the corresponding genes available in each dataset. Tumors were split into two groups according to the difference between the correlation obtained for the CpG-ODN treatment and the control condition, with the average used as threshold. Kaplan-Meier curves indicate the survival probability for ovarian cancer patients (panel A), breast cancer patients who received adjuvant systemic chemotherapy (panel B), and breast cancer patients who did not receive any systemic treatment (panel C). Patients with a greater correlation to the CpG-ODN compared to the untreated condition showed better outcome. Black curve: “CpG-like” patients; Gray curve: “CpG-untreated-like” patients.

**Figure 6. Kaplan-Meier plot of percent survivors over time among IGROV-1 ovarian tumor-bearing athymic mice.** Mice were treated i.p., starting from 8 days after tumor cell injection, with CpG-ODN (20 μg/mouse, 5 days/week for 4 weeks), cisplatinum (DDP, 3 mg/Kg i.p., once per week for 4 weeks) or both. Control mice received saline. Saline-treated mice (open circle); CpG-ODN-treated mice (open diamond), cisplatin-treated mice (filled triangle); CpG-ODN plus cisplatin-treated mice (filled square). Experimental groups consisted of 8-10 mice group.
Figure 4

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Figure 5

A. Overall survival (months)

B. Relapse-free survival (months)

C. Overall survival (months)

D. Relapse-free survival (months)
Figure 6
CpG-ODN and Cisplatin (DDP)

Days after cell injection

Percent survival