RESEARCH COMMUNICATION

Combination of IL-24 and Cisplatin Inhibits Cervical Cancer Growth in a Xenograft Nude Mice Model

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Abstract

IL-24/mda-7 delivery augments the sensitivity of several tumor types to cisplatin but the underlying mechanism(s) are unclear. Here, we used a cervical cancer xenograft model in nude mice to further elucidate the interaction between IL-24 and cisplatin. Nude mice were inoculated subcutaneously in the left axilla with Hela cells and randomly grouped into 5 treatment schedules: PBS (I); pDC316 vector (II); pDC316-hIL-24 (III); cisplatin (IV); and pDC316-hIL-24 combined with cisplatin (V). Groups III, IV and V showed significant reduction at mean tumor weight by 43%, 50% and 72%, respectively, after 4 weeks in comparison to thePBS and vector control groups. Mitotic counts in groups III, IV and V were also significantly reduced and expression of tumor suppressor gene nm23-H1 protein was significantly higher in groups III and V than in the cisplatin (IV), PBS (1), and vector (II) cases. The cisplatin group exhibited significantly greater weight loss than the other four groups. The mean weight loss of the combined group, while significantly more than in the controls and the IL-24 group, was significantly less than that of the cisplatin group. The IL-24 group and the combined therapy group exhibited enhancing effects on the tumor suppressor gene nm23H1expression.

Keywords: IL-24 - recombinant vector - cervical carcinoma - gene therapy - cisplatin - nm23H1

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Introduction

Cervical cancer (CC) was worldwide estimated at 530,000 cases of incidence and approximately 275,000 cases of mortality in 2008 (Arbyn et al., 2011) despite efficient screening methods and standard treatment including surgery, radiation, and chemotherapy such as cisplatin or 5-fluorouracil (5-FU) (Borowsky et al., 2005). Although chemotherapy initially showed 26-45% improvement in survival of CC patients, a retrospective study suggests that the benefits depend on the initial stage of CC (Borowsky et al., 2005). Side effect profile of cisplatin includes myelosuppression, renal toxicity, hearing loss, and tinnitus in 25-30% of patients, and marked nausea and vomiting in most patients whereas those of 5-FU also include nausea, vomiting, leukopenia, mental loss, seizures, excessive lacrimation, cardiovascular abnormalities, and hair loss. These serious side effects associated with cisplatin and 5-FU and the development of resistance indicate that additional strategies for treatment of cervical cancer are warranted.

Interleukin 24 (IL-24), initially discovered as melanoma differentiation associated gene-7 (mda-7), mediates significant anti-tumor activity and is now classified as a member of the IL-10 family. IL-24 has been effectively delivered to tumors with gene therapy by using replication-incompetent adenoviral vectors in cultured cells, several animal models, and a phase I clinical trial by multiple groups (Emdad et al., 2009). It induces apoptosis or toxic autophagy in many tumor types including breast cancer, glial blastoma, lung cancer, ovarian cancer, and liver cancer, but not in normal cells (Gopalkrishnan et al., 2004; Su et al., 2005; Gupta et al., 2006; Dash et al., 2010; Yacoub et al., 2010). In addition, IL-24 also stimulates an antitumor immune response, suppresses angiogenesis, and sensitizes cancer cells to radiation, chemotherapy, and antibody-mediated cytotoxicity (Emdad et al., 2009). It was reported that IL-24 may promote the survival of chronic lymphocytic leukemia B cells and wortmannin, a phosphatidylinositol 3 kinase inhibitor, can enhance IL-24-induced apoptosis and autophagy in these cells (Yang et al., 2010). Splice variants of IL-24 and their tissue specificity may account for some of the variability of responses (Whitaker et al., 2011).

IL-24 inhibits the migration and invasion of human cervical cancer cell lines in vitro (Shi et al., 2007). It can also inhibit growth and induce apoptosis of the cervical carcinoma Hela cells in vitro (Wu et al., 2009). However, its potential in augmenting treatment options for cervical cancer in vivo requires further studies. Since IL-24 can augment sensitivity of tumors to various chemotherapeutic agents in other types of tumors, our study investigated whether a combined treatment with IL-24 and cisplatin improved efficacy in comparison to the single modalities. Here, we compared the effect of combined IL-24 and cisplatin treatment on tumor growth, morphology,

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and nuclear division to each modality and control in a xenograft animal model of human cervical carcinoma. Because low nm23-H1 expression has correlated with cisplatin resistance in human breast, ovarian, and esophageal squamous carcinoma (Iizuka et al., 1999), we chose to assess the effect of the treatments on expression of nm23-H1 (nonmetastasis 23, H1 isoform), a marker associated with metastatic aggressive melanomas and breast carcinomas (Freije et al., 1998). Furthermore, positive expression of nm23 was negatively correlated with higher clinical stages, pathological grade, and lymph node metastasis in cervical cancer patients (Luo et al., 2004).

Materials and Methods

Vectors and reagents

The shuttle vector containing human IL-24 target gene fragment, pDC316 (pDC316-hIL-24) and empty vector pDC316 were purchased from Vector Gene Technology (Beijing, China).Transfection reagent, Lipofectamine 2000 was purchased from Invitrogen. Cisplatin was purchased from Qilu Pharmaceutical (Jinan,Shandong province,China). Rabbit anti-nm23-H1 polyclonal antibodies were purchased from Boster Bio-engineering (Wuhan, China). Endotoxin-free plasmid extraction and purification kits were purchased from Solarbio (Beijing, China).

Cell culture

Human cervical carcinoma cell line, Hela cells, was purchased from Cell center in Peking Union Medical College. Hela cells were cultured in DMEM high glucose medium containing 10% FBS in 37°C incubator at 5% CO_2.

Use and care of animal

Specific pathogen-free (SPF) female nude BALB/c mice (3 weeks old, 13~15g) were purchased from slrc laboratory animal center (Shanghai, China). Mice were maintained in pathogen-free conditions and used for study in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All surgical procedures and care administered to the animals were approved by the institutional ethics committee.

Tumor xenograft mouse model

Hela cells at logarithmic phase were harvested with EDTA. HeLa cells (100 μL; $2x10^6$ cells) were injected subcutaneously into the left axilla in 4-week-old nude BALB/c mice. Fourteen days later, nude mice developed subcutaneous xenograft tumors (>5×5mm). The tumor-bearing nude mice were randomized into the following 5 treatment groups (n=6) as summarized in Figure 1: (I) PBS buffer control group, intratumoral injection: 100μL of PBS buffer was injected at day 3, 6, 9, 12, and 15. (II) Empty vector treatment group, intratumoral injection: 100μL of empty vector pDC316 (100 mg/L) and 25μL of Lipofectamine 2000 for transfection were mixed in advance and injected at day 3, 6, 9, 12, and 15. (III) IL-24 treatment group, intratumoral injection: each tumor was injected at multiple sites with a mixture of 125μL of pDC316-hIL-24 (10mg/L) and Lipofectamine 2000 (4:1, v/v) for transfection at day 3, 6, 9, 12, and 15. (IV) Cisplatin group, peritoneal injection: 0.5mL of cisplatin at 5 mg/kg were injected at day 0, 1 and 2. (V) Combinational treatment group, intratumoral injeaction for IL-24 and peritoneal injection for cisplatin: cisplatin (0.5 ml, 2.5 mg/kg) was administered 3 times as aforementioned. After 24 hrs, each tumor was injected with IL-24 as indicated in group 1. Tumor size (length and width, L and W, respectively) was measured with a caliber every 3 days. Size of tumor was calculated as $V(mm^3) = L \times W2/2$. Two weeks after completion of treatments, mice were sacrificed via cervical dislocation and weighed. Final tumor dimensions and weights were measured. Tumor suppression rate (%) equaled [average tumor weight of control group - average tumor weight of treatment group/ average tumor weight of control group] ×100%.

hIL-24 expression in tumor by RT-PCR

Amplification of human IL-24 included the Hind III and SalI restriction enzyme sites in the upstream and downstream primers, respectively. Primer sequences were as follows: upstream: 5'-GCCAAGCTTATGAATTTTCAACAGAGG-3', downstream: 5'-GCCGTCGACCTAGA-GCTTGTAGAATTT-3'. Amplification conditions were denaturation at 94°C for 2 min, and 32 cycles of 94°C denaturation for 20 seconds, 58°C annealing for 20 seconds, 72°C extension for 20 seconds, followed by 72°C extension for another 8 min. PCR products were separated by gel electrophoresis and the size was identified by standard markers. Generation and identification of pDC316-hIL-24: the PCR amplified and ligated product was sequenced by Zhongshan Biotech (Beijing).

Pathological observation of tumor tissue

Tumor sizes, color, and necrotic section of tumor were recorded. Tumor tissue was sectioned following formalin fixation and wax embedding. Hematoxylin and Eosin (HE) staining, mitotic figure count, tumor cell density, and nuclear atypia were observed under optical microscope with 400X magnification and semi-quantified by experienced pathologists in a double blind manner. (1) Mitotic count: ten distinct areas with the highest density on each slide were counted and averaged (Zhang and Xu, 1996). (2) Cellular atypia was classified into high and low group, depending on nuclear size, morphology, nuclear-cytoplasm ratio and nuclear staining level. Ten non-necrotic areas at high resolution were assessed for

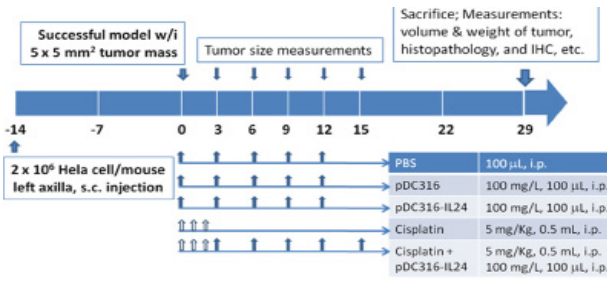

Figure 1. Treatment Groups, Dosages, and Schedules in Cancer Xenograft Model in Nude Mice

Table 1. Mean Tumor Volumes of the Five Groups During Treatment

Day	Tumor volume (mm^3)					P-value
	Control	Vector	IL-24	Cisplatin	Combined	
0	72.6±6.24	72.5±7.9	71.2±5.7	69.7±5.2	69.5±3.1	0.819
3	123.2±11.7	123.1±8.2	119.9±7.2	111.6±8.5	118.0±6.4	0.153
6	212.0±19.2	204.0±18.2	199.3±10.5	181.1±13.5†	168.3±10.9†‡§	<0.001*
9	363.0±43.3	365.0±46.5	337.3±29.0	281.7±30.3†‡	208.3±49.2†‡§‖	<0.001*
12	594.3±62.8	592.8±56.1	437.3±60.9†‡	389.8±49.4†‡	238.2±42.4†‡§‖	<0.001*
15	845.5±110.0	835.3±105.7	486.4±70.1†‡	449.6±52.4†‡	246.2±42.4†‡§‖	<0.001*

Data were expressed as Mean±SD. The differences among five groups were conducted by one way ANOVA; *P-value<0.05, indicated a significant difference among five groups; †P-value<0.05, compared with control group; ‡P-value<0.05, compared with Vector group; §P-value<0.05, compared with IL-24 group; ‖P-value<0.05, compared with group

Table 2. Mean Weight of Mice, Mean Tumor Weights andMean Tumor Inhibition Rates in the Five Groups on Day 29

	Control	Vector	IL-24	Cisplatin	Combined	P-value
Total weights (g)	23.5±1.2	23.1±0.8	22.3±0.8	15.9±0.9†‡§	19.4±1.3†‡§‖	<0.001*
Tumor weight (g)	4.7±1.0	4.6±1.1	2.7±0.7†‡	2.6±0.5†‡	1.3±0.6†‡	<0.001*
Tumor inhibition rate	-	3.7±0.2	42.8±0.2‡	50.3±0.1‡§	71.7±0.1‡§‖	<0.001*

Data were expressed as Mean±SD. The differences among five groups were conducted by one way ANOVA. N=6/group; *P-value<0.05, indicated a significant difference among five groups; †P-value<0.05, compared with control group; ‡P-value<0.05, compared with Vector group; §P-value<0.05, compared with IL-24 group; ‖P-value<0.05, compared with group

each slide. (3) Tumor cell density: 10 non-necrotic fields of high resolution under microscope for each slide were classified as 'dense (+)": tumor cells were aggregated with a lot of intercellular gap; "very dense (++)": tumor cells were tightly aggregated with partial intercellular gap; and 'extremely dense (+++)": tumor cells aggregated in piles with very little intercellular gap.

Immunohistochemical identification of nm23H1 expression

Tumor tissues were fixed with formalin, embedded in wax, sectioned, and stained with Hematoxylin and Eosin (HE), as described in the instructions of the reagent kit (rabbit anti-nm23H1polyclonal Ab in situ hybridization kit, BA0515, Boster, Wuhan, China). Known positive slide was used as positive control. PBS buffer replacing the primary antibody was used as a negative control. At low microscopic resolution (100X), evenly stained areas in tumor were located. At high microscopic resolution (400X), 3 fields of cells with positive staining were collected and analyzed by BI2000 medical image analysis system: grayscale value of positive cells was recorded. Here the grayscale reflects the intracellular heterochromatic granules of the content in a reversed manner. The smaller the grayscale value, the deeper staining of heterochromatic granule. In the other word, the higher the grayscale value is, the less positive signals are detected.

Statistical analysis

The data were analyzed by SPSS 13.0 statistic software. The results were expressed as means and standard deviations (SD). Comparisons of mean tumor volumes, tumor weights, nuclear division and nm23H1 expression among five groups were conducted as one way analysis of variance (ANOVA). When a significant difference appeared, multiple comparisons were performed using the Bonfferoni procedure with type-I error adjustment. The level of significance was set at 0.05. Statistical analyses were performed by SAS 9.1 (SAS Institute Inc., Cary, NC).

Results

Tumor growth

As shown in Table 1, mean tumor volumes of all groups increased in a time-dependent manner. Mean tumor volumes of the PBS and vector control groups were similar throughout the experiment. Mean tumor volumes of the IL-24, cisplatin, and combined treated groups were significantly smaller on the 12th and 15th day compared to that of the PBS and vector controls ($p<0.001$, respectively). No statistical difference was observed between the IL-24 and cisplatin groups. The combined group had significantly smaller mean tumor volume on days 9, 12, and 15 than the controls and the single modality groups did.

On day 29, mean tumor weights of the IL-24, cisplatin, and the combined groups were significantly lower than that of the PBS control and vector groups ($p<0.05$, respectively). Mean tumor volumes and weights in the IL-24 and cisplatin groups showed no significant difference. As compared to the vector group, mean tumor growth in the IL-24 (42.8%), cisplatin (50.3%) and combined IL-24 and cisplatin groups (71.7%, Table 2) remained significantly inhibited 2 weeks after treatment compared to the PBS and vector control groups.

Weight loss and condition of nude mice

Before treatment, the mean body weights of nude mice in each group were not statistically different ($F=0.174$, $P>0.05$). By day 6, the cisplatin-treated nude mice were observed to become gradually less active, their food intake decreased, and they had weight loss significantly more than PBS control group. Nude mice in the other four groups showed good spirits and normal intake of food and water. At the end of treatment (~ 15 days), the mean body

Table 3. Nuclear Division and the Expression of Nm23H1 in the Five Groups

	Control	Vector	IL-24	Cisplatin	Combined	P-value
Nuclear division (number/HP)	10.3±1.2	10.0±1.4	4.7±1.0†‡	6.0±0.9†‡	3.2±1.2†‡‖	<0.001*
nm23H1 expression	175.0±4.5	175.9±4.5	148.0±8.3†‡	171.9±9.8§	143.6±9.2†‡‖	<0.001*

Data were expressed as Mean±SD. The differences among five groups were conducted by one way ANOVA. There're 6 mice in each group; *P-value<0.05, indicated a significant difference among five groups; †P-value<0.05, compared with control group; ‡P-value<0.05, compared with Vector group; §P-value<0.05, compared with IL-24 group; ‖P-value<0.05, compared with group

weight differences among the groups were statistically significant (F=58.741, P<0.001) (data not shown). Mean body weight was reduced significantly in the cisplatin group, while mean body weights of the control and IL-24 groups showed no significant difference. The combined group exhibited a lesser degree of the body weight loss compared to the cisplatin group (Table 1). At the end of the experiment (~29 days), the mean body weights in the PBS control, empty vector, and IL-24 treatment groups were not statistically different (Table 2).

On day 29, there were significant differences between the mean tumor weights of most of the groups (F=19.832, P<0.001). As expected, the mean tumor weights of the PBS and vector controls were similar. The average tumor weights of the three treatment groups were significantly smaller than that of the two control groups (P<0.05). The mean tumor weight of the combinational treatment group (group V) was the lightest, followed by cisplatin group (IV) and IL-24 group (III). Similarly, suppression of tumor growth in treatment groups III, IV, and V were 42.8%, 50.3%, and 71.7%, respectively (Table 2). The combination treatment group (group V) showed the greatest anti-tumor effect (P<0.05).

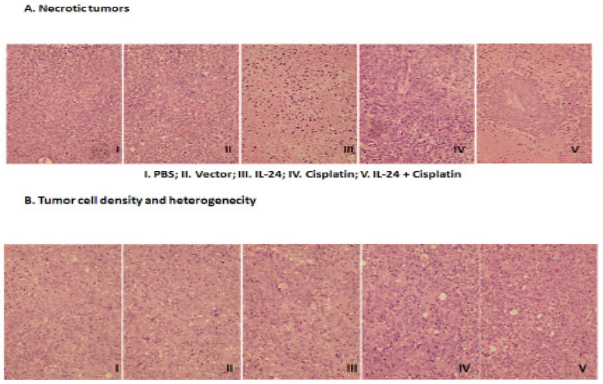

Figure 2. Tumor Morphology. A. Tumor Cell Density on Day 29. B. Nm23-H1 Staining on Day 29. Representative Sample from Each of the 5 Groups. I. PBS group; II. Vector group; III. IL-24 group; IV. group; V. Combined therapy (IL-24 +) group

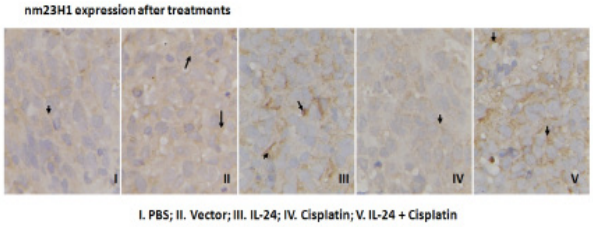

Figure 3. Expression of Nm23H1 Visualized by IHC in the Control and Treatment Groups. The nm23H1 protein was expressed in the cytoplasm of cervical carcinoma cells and less in the nucleus (arrow indicated). Quantitative analysis was shown in Table 3

Tumor morphology

Tumors in the vector and PBS control groups were larger, red with obvious blood supply on the surface and showed obvious infiltration to the surrounding tissue and skin (Figure 2A-I, 2A-II). Tumors of the IL-24-treated group were light red with few observable blood vessels and had local necrosis in the tumor section (Figure 2A-III). Tumors of the cisplatin group were red with multiple, obvious blood vessels on the surface and evidence of necrosis (Figure 2-IV and 3A-IV). Tumors in the combined treatment group had a solid texture, few visible blood vessels, grayish color, and an edge distinct from surrounding tissue which provided an easy separation (Figure 2A-V).

Cell morphology

H&E staining of the tumor tissue sections in the empty vector and PBS control groups showed a dense configuration of the cancer cells (Figure 3B-I, 3B-II). In the IL-24, cisplatin and combinational treatment groups, the cancer cell density was decreased to different extents, though didn't show statistical significance. As shown in Figure 3B, cancer cells in the tumors of the PBS and empty vector control groups showed significant nuclear atypia. The cancer cells of the IL-24, cisplatin and combined treatment groups showed nuclear atypia, although less than the controls.

Mitotic cells and nm23-H1 expression

The IL-24, cisplatin, and combined groups showed significantly less mitotic cells than the PBS control and vector groups did. IL-24 and the cisplatin groups showed no significant differences in mitotic counts (Table 3).

Positive nm23-H1 staining was distributed mostly in the cytoplasm. As shown in Figure 4, most nm23H1 was expressed in the cytoplasm of cervical carcinoma cells and less in the nucleus. The gray scale value of nm23H1 positive cells were statistically different between groups (F=102.303, p<0.001). Although groups of PBS, vector, and cisplatin showed similar intensity of nm23H1 positive cells (P>0.05) (Table 3), the number of nm23H1 cells in the IL-24 and the combined groups was significantly less than in the controls.

Discussion

In recent years, chemotherapy for cervical cancer has become a part of standard care. Chemotherapeutic resistance of cervical cancer reduces the efficacy of the current standard of care. Increasing the sensitivity of cervical cancer to chemotherapeutic agents and identifying

viable adjunct therapies for cervical cancer may improve the outcomes of treated patients.

Currently, cisplatin is a common therapeutic agent for cervical cancer, and it mainly forms cisplatin-DNA adducts that interrupt DNA replication. Recently, a trial showed that once weekly administration of cisplatin was equally efficacious with fewer side effects than thrice weekly administration (Lee et al., 2011). IL-24 has significant direct and indirect anti-tumor activity against numerous cancers and has shown efficacy in clinical trials against metastatic melanomas (Emdad et al., 2009). IL-24 toxicity on normal cells has not been reported (Su et al., 2005). Here, we investigated the potential of IL-24 to augment cisplatin-induced cytotoxicity of human cervical cell line in a xenograft tumor model in nude mice.

Combined treatment of cisplatin and IL-24 for cervical cancer xenograft in nude mice.

In this study, treatment with cisplatin, IL-24, and the combined cisplatin-IL-24 group significantly inhibited tumor growth compared to the PBS and the vector control groups. Cisplatin treatment exhibited comparable anti-tumor efficacy as IL-24 single treatment. The mean tumor volume of the combined cisplatin-IL-24 treatment group was significantly smaller than single modality groups (IL-24 or cisplatin). The combined therapy had reduced tumor growth by greater than 70%, suggesting that efficacy of the combined treatment was superior to single modality treatment. Historically, anti-tumor efficacy of cisplatin is dose dependent. Although the dosage of cisplatin in the combined treatment group was half the cisplatin dose in the cisplatin single modality group, the efficacy was significantly greater. While monitoring of the effects of additional doses and combinations are needed to elucidate the type of drug interaction between IL-24 and cisplatin in cervical cancer, IL-24 has previously increased the sensitivity of breast tumors to chemotherapy including cisplatin (Emdad et al., 2009). Furthermore, cisplatin but not IL-24 treatment had significantly reduced the mean weight gain of the mice. The combination therapy group had a greater weight gain than the cisplatin single modality group but not the IL-24 group. Whether the reduced toxicity arose from the use of half the dose of cisplatin of the single modality group, the presence of IL-24, or both requires further studies.

Histopathological analysis revealed that the cisplatin, IL-24, and the combined therapy significantly reduced tumor cell density and mitotic figure count, consistent with their effects on tumor growth. Cellular atypia of cancer cells characterizes the morphologic differences between cancer cells and normal tissues, such as bazzard nucleus shape, darkened chromatin, increased nuclear-cytoplasm ratio and inconsistent nucleus sizes. These properties indicated tumorigenesis at different stages during differentiation. Because we found no statistical differences between the groups, we suggest that cisplatin and IL-12 treatment do not affect tumor cell differentiation.

nm23-H1 suppresses metastases at least in part by down-regulating expression of EDG2 (endothelial differentiaon, lysophosphatidic acid G-protein-coupled receptor) (Horak et al., 2007). Mutation or low expression of nm23H1 has been highly associated with tumor metastasis in gastric cancer, lung cancer, breast cancer, esophageal cancer and cervical cancer (Freije et al., 1998), but positively correlated with liver metastasis of gastric cancer and colorectal cancer (Freije et al., 1998). Recently, Wang et al showed that cervical cancer patients with positive nm23-H1 but negative lipocalin-2 expression had the highest probability of recurrence and the lowest overall survival (Wang et al., 2011). Here, we observed that the IL-24 treated group and the combination group had significantly higher expression of nm23-H1 than the control, empty vector and cisplatingroups. These results suggested that IL-24 increased the expression of nm23-H1. Moreover, observation of tumor tissues indicated that the infiltration to the surrounding tissue and skin of the xenograft tumor was significantly reduced in co-treatment and IL-24 treatment groups. These data are consistent with IL-24 promoting expression of the metastasis suppressing gene nm23H1, which might be one of the anti-tumor mechanisms for IL-24. Furthermore, since cisplatin resistance is associated with lower nm23-H1 expression (Iizuka et al., 1999), the observation that IL-24 augments nm23-H1 expression may provide a mechanism for enhanced sensitivity to cisplatin. In conclusion, IL-24 reduced tumor growth, and provided a significant anti-tumor activity with cisplatin. IL-24 may have also reduced the effects of cisplatin on weight gain in the combination. However, further mechanistic studies are needed to elucidate signal transduction pathway(s) employed by IL-24 to reduce tumor growth and improve sensitivity to cisplatin and other chemotherapeutic drugs.

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