Resistance and perspectives in soft tissue sarcomas
Komdeur, Rudy

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2003

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Chapter 5

Expression of multidrug resistance–associated proteins in rhabdomyosarcomas before and after chemotherapy: the relationship between lung resistance–related protein (LRP) and differentiation

J.W. Klunder, R. Komdeur, W.T.A. van der Graaf, E.J.S.M. de Bont, H.J. Hoekstra, E. van den Berg, W.M. Molenaar

*Human Pathology* 2003;34:150-155
Abstract

Rhabdomyosarcomas generally respond well to chemotherapy, and the residual lesions often are better differentiated than their primaries. This phenomenon may be explained by selective multidrug resistance (MDR) of differentiated tumor cell populations. We assess the role of MDR proteins in chemotherapy-induced differentiation in rhabdomyosarcomas in a clinical setting. Paraffin-embedded samples of 13 pairs of primary untreated rhabdomyosarcomas and their residual, recurrent, or metastatic lesions after chemotherapy were assessed for expression of MDR proteins, including P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP1) and lung resistance-related protein (LRP). Expression was semiquantitatively scored based on the percentage of isolated immunoreactive tumor cells as follows: 0, negative; 0.5, <5%; 1, 5% to 25%; 2, 26% to 50%; 3, 51% to 75%, and 4, >75%. All specimens after chemotherapy, except the late recurrences, were better differentiated than their primary, untreated specimens. P-gp or MRP1 expression did not change significantly, but LRP expression increased significantly after chemotherapy. In both untreated and treated samples, LRP was expressed primarily in differentiated cells. The findings indicate that the in vivo expression of LRP, but not of P-gp and MRP1, is induced by chemotherapeutic treatment in rhabdomyosarcomas. The preferential expression of LRP in differentiated cells and the subsequent more extensive expression after chemotherapy suggest that LRP plays a role in therapy-induced differentiation.

Introduction

Rhabdomyosarcomas generally respond fairly well chemotherapy, especially in children.\(^1\)\(^-\)\(^3\) The residual lesions often show remarkable morphologic differentiation with increased expression of muscle-specific intermediate filament, desmin, and other myogenic markers.\(^4\)\(^-\)\(^8\) Based on comparisons of residual tumors after chemotherapy with their primaries, it has been suggested that chemotherapy selectively eliminates undifferentiated tumor cells and possibly induces further differentiation in the remaining, partially differentiated cells.\(^8\) Studies in rhabdomyosarcoma cell lines have further demonstrated the potential of chemotherapeutic agents to induces differentiation.\(^9\)\(^-\)\(^13\)
Response to chemotherapy may be influenced by multidrug resistance (MDR), which reflects the insensitivity of tumor cells to various structurally unrelated natural chemotherapeutic agents. This quality may be already present in chemotherapy-naive tumor cells, and also may be acquired under the pressure of chemotherapeutic treatment. A number of proteins have been identified as possibly playing a role in conveying MDR. Among these are the drug efflux pump proteins P-glycoprotein (P-gp) and multidrug resistance–associated protein 1 (MRP1) and the major vault protein lung resistance–related protein (LRP). The incomplete response of rhabdomyosarcomas to chemotherapy raises the question as to whether heterogeneity in the expression of MDR proteins plays a role in selective sensitivity of different tumor cell populations. The present study analyzed the expression of these proteins in 13 pairs of primary rhabdomyosarcomas and their residual, metastatic, or recurrent lesions after chemotherapy in pediatric and adult patients.

**Patients and Methods**

**Patients.** Cases were selected based on the following criteria: (1) a histologic diagnosis of rhabdomyosarcoma, (2) sufficient paraffin material for both the primary and follow-up specimens, and (3) intervening treatment with chemotherapy. This resulted in 9 pairs of primaries and residual tumors, 2 pairs of primaries and metastases, and 2 pairs of primaries and local recurrences (Table 1). In cases $k$ and $l$, the primary tumors were completely resected before chemotherapy (by orchidectomy in case $k$ and by resection of a nasal polyp in case $l$), but metastases developed in the retroperitoneum and bone marrow, respectively. In cases $m$ and $n$, the primary tumor areas were resected after chemotherapy, but no viable tumor was present. Local recurrences occurred after 17 and 42 months, respectively.

**Histology.** All cases were reviewed histologically on hematoxylin and eosin-stained sections and with desmin stains. The tumors were further classified as embryonal, alveolar, or pleomorphic according to the criteria specified by Enzinger and Weiss. The relative numbers of primitive undifferentiated cells, of large “rhabdomyoblasts” with ample cytoplasm, and of strap cells with or without cross-striations were compared between primary tumors and the corresponding follow-up material, as was the extent of desmin immunoreactivity.
Table 1. Patient data.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yrs)/Sex</th>
<th>Site</th>
<th>Type</th>
<th>Primary treatment</th>
<th>Follow-up material</th>
<th>Interval (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>13/F</td>
<td>Ovary</td>
<td>E</td>
<td>Chemotherapy*</td>
<td>Residual</td>
<td>18</td>
</tr>
<tr>
<td>b</td>
<td>16/F</td>
<td>Perineum</td>
<td>E</td>
<td>VAC</td>
<td>Residual</td>
<td>23</td>
</tr>
<tr>
<td>c</td>
<td>30/F</td>
<td>Palate</td>
<td>E</td>
<td>VAC+RT</td>
<td>Residual</td>
<td>17</td>
</tr>
<tr>
<td>d</td>
<td>40/F</td>
<td>Retroperitoneum</td>
<td>E, epirubicin</td>
<td>Residual</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>e</td>
<td>9/F</td>
<td>Retroperitoneum</td>
<td>E</td>
<td>VAC + RT</td>
<td>Residual</td>
<td>18</td>
</tr>
<tr>
<td>f</td>
<td>14/M</td>
<td>Tonsil</td>
<td>E</td>
<td>VAC + RT</td>
<td>Residual</td>
<td>30</td>
</tr>
<tr>
<td>g</td>
<td>0/M</td>
<td>Nose</td>
<td>E</td>
<td>VAC</td>
<td>Residual</td>
<td>8</td>
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<tr>
<td>h</td>
<td>7/F</td>
<td>Urinary bladder</td>
<td>E</td>
<td>VAC</td>
<td>Residual</td>
<td>17</td>
</tr>
<tr>
<td>j</td>
<td>10/M</td>
<td>Retroperitoneum</td>
<td>E</td>
<td>VAC +</td>
<td>Residual</td>
<td>27</td>
</tr>
<tr>
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<td>Spermatic cord</td>
<td>E</td>
<td>CT</td>
<td>Metastasis</td>
<td>58</td>
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<tr>
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<td>39/F</td>
<td>Nose</td>
<td>E</td>
<td>EVI*</td>
<td>Metastasis</td>
<td>20</td>
</tr>
<tr>
<td>m</td>
<td>19/F</td>
<td>Foot</td>
<td>A</td>
<td>EVI</td>
<td>Recurrence</td>
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</tr>
<tr>
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<td>Thigh</td>
<td>E</td>
<td>VAC + RT</td>
<td>Recurrence</td>
<td>181</td>
</tr>
</tbody>
</table>

Abbreviations: M, male; F, female; E, embryonal; A, alveolar; VAC, vincristine/actinomycin D/cyclophosphamide; EVAIA, etoposide/vincristine/actinomycin D/ifosfamide/adriamycin; EVI, epirubicin/vindesine/ifosfamide; RT, radiotherapy

* Further details on chemotherapy not available

Expression of multidrug-resistant proteins. P-gp, MRP1, and LRP expression was assessed by immunoperoxidase procedures. Samples were deparaffinated in xylene and alcohol. Antigen retrieval was achieved by heating the samples at 115°C under pressure (10 psi) in an autoclave for 3 cycles of 5 minutes each. The monoclonal C494 (concentration 120 µg/mL, dilution 1:200; Signet Laboratories, Dedham, MA), which recognizes P-gp, was used. For MRP1, noncommercial monoclonal antibody MRPr1 (concentration 20 µg/mL, dilution 1:15; kindly provided by Prof. Dr. R.J. Scheper, Department of Pathology, Free University Medical Center, Amsterdam) was used. For LRP, an anti-LRP monoclonal antibody (concentration 250 µg/mL, dilution 1:400; Transduction Laboratories, Los Angeles, CA) was used. The samples were incubated with the primary antibody-containing dilution at room temperature for 1 hour. For each sample, a peroxidase-conjugated secondary antibody identified the binding of the primary antibody. Diaminobenzidine-tetrahydrochloride (Sigma, St. Louis, MO) in phosphate-buffered saline was used as the chromagen. Samples were counterstained with hematoxylin. Liver, lung, and colon tissue served as positive controls for...
P-gp, MRP1, and LRP expression, respectively. The expression was estimated and scored based on the percentage of single immunoreactive tumor cells as follows: 0, no immunoreactivity; 0.5, <5%; 1, 5% to 25%; 2, 26% to 50%; 3, 51% to 75%; and 4, 76% to 100%.

Statistics. The statistical significance of the changes in expression of each MDR protein in tumor pairs was assessed using the Wilcoxon signed-rank test.

Results

Patients. The study group comprises 13 patients (eight pediatric and five adult patients). The relevant clinical data are given in Table 1.

Histology. All except one of the tumors that fulfilled the selection criteria appeared to be of the embryonal type. Only case m was of the alveolar type. All cases expressed desmin. Comparing primary tumors and their corresponding residual tumors or metastases, all specimens after chemotherapy showed an increase in differentiation compared with their primaries, as well as increased desmin expression (Figure 1A and B). This was not the case for the late recurrences of cases m and n.

MDR protein expression. P-gp was expressed in all but one primary tumors and was scored as 3 or 4 in nine of 13 cases. In four cases, including the only negative primary case and both recurring cases, expression increased after chemotherapy. In four cases, P-gp expression decreased, whereas in the remaining five cases, expression was similar in the prechemotherapy and postchemotherapy material (Figure 2A). The changes in P-gp expression were not statistically significant.

MRP1 was expressed in all except two primary tumors and was scored as 3 or 4 in seven cases. In the follow-up material of three cases, including both late recurrences, expression increased strongly after chemotherapy. A few positive cells were found in one metastasis of one negative primary tumor. In five cases, expression decreased, whereas in the remaining four cases, expression remained similar to that before chemotherapy (Figure 2B). The changes in MRP1 expression were not statistically significant.

In contrast to P-gp and MRP1 expression, LRP expression was very limited in the primary tumors and was scored as 4 in only one case. Two cases were negative, and the remaining cases showed only a limited
Figure 1. Changes in desmin and LRP expression after chemotherapy. Desmin expression in case j before (A) and after (B) chemotherapy, and LRP expression in case e before (C) and after (D) chemotherapy. Arrows indicate differentiated LRP expressing tumor cells.
Figure 2. Pairwise comparison of P-gp (A.), MRP1 (B.), and LRP (C.) expression in primary rhabdomyosarcomas and their residual lesions, metastases (met.) and recurrences (rec.) after chemotherapy.
MDR proteins in rhabdomyosarcomas before and after chemotherapy

number of immunoreactive cells. However, LRP expression was most prominent in better-differentiated tumor cells (Figure 1C and D). After chemotherapy, five of nine residual lesions, both metastases, and one of the two recurrences showed (strongly) increased LRP expression (Figure 2C). Only one residual tumor (case b) showed a slight decrease; all of the other postchemotherapy specimens showed expression similar to that of their prechemotherapy counterparts, one of which already showed maximal LRP expression before chemotherapy. The changes in LRP were significant in a pairwise analysis of the whole group (P = 0.015), as well as after exclusion of the late recurrences (P = 0.021).

Discussion

Chemotherapy-induced differentiation in rhabdomyosarcomas is now a well-established phenomenon, documented in histologic samples as well as in cell lines. However, the mechanisms involved and the clinical significance are still only partially understood. The “differentiated” cells that remain after chemotherapy appear to represent a tumor subpopulation that is resistant to chemotherapy in an otherwise very chemosensitive tumor. This raises the question as to whether MDR may play a role in selective “protection” of better-differentiated tumor cells. To explore this notion, the present study compared the expression of MDR proteins in 13 pairs of primary, untreated rhabdomyosarcomas and follow-up material obtained after chemotherapy. All except two specimens obtained after chemotherapy showed morphologic “maturation” and increased desmin expression, as previously described. The two cases in which no further differentiation was observed represent late local recurrences in cases in which the primary tumor areas were resected after chemotherapy, but showed complete remission. It may well be that in these cases, primarily chemosensitive clones reemerged.

After chemotherapy, P-gp and MRP1 expression remained essentially unchanged, whereas LRP expression increased significantly. These findings suggest P-gp and MRP1 possibly play a role in primary chemoresistance in rhabdomyosarcomas, whereas LRP may be involved in chemotherapy-induced MDR. Studies on chemotherapy-induced changes in MDR in soft tissue sarcomas are rare, and most deal with mRNA, not with protein expression as in the present study. One in vivo study demonstrated up-regulation of the mRNA of MDR1 (the gene for P-gp) in five patients after isolated lung perfusion with doxorubicin for lung
Other studies have reported higher MDR1 mRNA in treated than in untreated rhabdomyosarcomas, as well as in neuroblastomas and pheochromocytomas and a change from P-gp negative to positive in one sarcoma. The study by Marchal et al. of the embryonal rhabdomyosarcoma cell line RD revealed chemotherapy-induced differentiation not accompanied by up-regulation of MDR1 mRNA. In contrast, another study by the same group showed up-regulation in two rhabdomyosarcoma cell lines, one of which was obtained after chemotherapy in vivo. Finally, one study that assessed changes in P-gp, MRP1, and LRP expression in soft tissue sarcomas after isolated limb perfusion with tumor necrosis factor alpha and melphalan found no consistent changes. More data are available on hematologic malignancies. In a series of more than 100 bone marrow biopsy samples from patients with plasma cell myeloma, P-gp was increased after treatment with doxorubicin and vincristine; in both acute myeloid and acute lymphoid leukemias, MRP (but not MDR1) mRNA was increased significantly after treatment, and P-gp function and expression were unchanged between diagnosis and relapse in blasts of 30 patients with acute myeloid leukemia. In 17 pairs of bone marrow biopsies of acute myeloid leukemia patients, LRP was increased at relapse compared with the pretreatment specimens. The increased LRP expression was accompanied by morphologic differentiation in the residual specimens. Interestingly, a relation between maturation and expression of major vault proteins (MVPs), the major component of LRP, was also recently established in human dendritic cells. Moreover, blocking of MVPs led to disturbed maturation and decreased viability of these cells. In a study of various tumor types, Izquierdo et al. found that LRP expression was higher in differentiated tumor types and present in a number of refractory tumors after chemotherapy, including two rhabdomyosarcomas. Recent studies by Kitazono demonstrated that LRP mRNA and protein increased after induced differentiation in a colon carcinoma cell line.

Although the same phenomenon has been observed in different malignancies, this does not necessarily mean that the same mechanisms are involved. Chemotherapy-induced differentiation of tumor tissue may be explained by selective destruction of undifferentiated tumor cell subpopulations, by direct induction of differentiation, or a combination of both of these mechanisms. Based on morphologic and immunohistologic observations, it has been suggested that both elimination of the most primitive, chemosensitive tumor cells and differentiation induction of committed cells occur. Several studies in rhabdomyosarcoma cell
MDR proteins in rhabdomyosarcomas before and after chemotherapy

lines have demonstrated the differentiation-inducing potential of antineoplastic agents. The studies by Marchal et al. further revealed that the predominance of differentiation induction or selective destruction may depend on the chemotherapeutic agent and/or dosage. Taken together, the earlier and current findings suggest heterogeneity in rhabdomyosarcomas with respect to both chemosensitivity and differentiation level. It is likely that the better-differentiated cells are more chemoresistant, which appears to be conveyed by expression of LRP (Figure 3).

The clinical relevance of the aforementioned findings remains to be established. Controversy exists with respect to the significance of tumor cell differentiation in relation to survival. No clear distinction has been made between residual tumors after chemotherapy and (late) relapses, which may represent different phenomena. In vitro studies have demonstrated both decreased proliferative activity and decreased tumorigenicity after treatment with Ara-C. A small series of tumor specimens obtained before and after chemotherapy found an apparent association between differentiation, decreased proliferative activity, and favorable prognosis. In view of the current findings, (pre)terminal differentiation induced by chemotherapy might increase LRP-related MDR, which should be taken into consideration when planning further treatment.

Figure 3. Relationship between differentiation, chemotherapy, and LRP expression. Small arrows indicate progressive morphologic differentiation from "primitive cells" to immature and mature "round rhabdomyoblasts" and "strap cells". D, desmin; −/+/+++ negative / moderately/ strongly positive. Large arrows indicate the presumed increasing or decreasing effects of chemotherapy compared with morphology.
References

MDR proteins in rhabdomyosarcomas before and after chemotherapy