Regression of Melanoma in a Murine Model by RLIP76 Depletion

Sharad S. Singhal, Yogesh C. Awasthi, and Sanjay Awasthi

Abstract
RLIP76/RALBP1 is a stress-responsive membrane protein implicated in the modulation of multiple cellular signaling pathways. It represents the predominant glutathione-conjugate transporter in cells, and our previous studies have shown that its inhibition by antibodies or depletion by short interfering RNA (siRNA) causes apoptosis in a number of cancer cell types. The present studies were designed to explore the potential clinical applicability of our previous observations by comparing the relative expression of RLIP76 in cancer versus normal cell lines and to determine whether depletion of RLIP76 activity can exert cancer-specific apoptosis. RLIP76 expression was found to be significantly greater in malignant cells compared to nonmalignant cells. Inhibition of RLIP76, using antibodies towards a cell surface epitope, or depletion of RLIP76 using either siRNA or antisense phosphorothioate oligonucleotides preferentially caused apoptosis in malignant cells. More importantly, in vivo studies showed that administration of RLIP76 antibodies, siRNA, or antisense oligonucleotides to mice bearing syngeneic B16 mouse melanoma cells caused complete tumor regression within 10 days. These findings strongly suggest that RLIP76 depletion by genetic approaches or inhibition by antibodies may be a clinically viable antineoplastic therapy, particularly for melanoma.

Introduction
Most presently available antineoplastic therapies for human malignancies are limited by the occurrence of significant normal tissue toxicities due to inherent relatively nonspecific genotoxic or signaling effects. Thus, attempts to improve antineoplastic therapies have focused on identifying targets which are preferentially expressed in cancer cells, which when inhibited, cause apoptosis in malignant cells although sparing cells of normal tissues. Delineation of differentially expressed signaling proteins that are responsible for unregulated growth and suppression of normal apoptotic pathways has led to the identification of numerous potential targets, which when inhibited should cause apoptosis preferentially in cancer cells while sparing normal cells. Some degree of success has been achieved in these endeavors, leading to the development of antibody drugs such as Rituxan (anti-CD20 antibody) for lymphoproliferative disorders (1–3), Herceptin (anti-Her-2/neu antibody) for breast cancer (4, 5), as well as small-molecule drugs including Gleevec (Bcr-Abl kinase inhibitor) for chronic myelogenous leukemia (6), and Tarceva (tyrosine kinase inhibitor) for a variety of solid tumors (7). The overall efficacy of these therapy is still limited because they are either effective only in a small fraction of patients with these malignancies, or due to clonal selection of cancer cells inherently refractory to these therapies. Thus, the development of new targeting molecules and the discovery of new targets remains a major objective in the development of oncological therapeutics.

RLIP76 (RALBP1) represents a novel potential target for cancer therapy not only because it represents a rate-controlling step in glutathione (GSH)-mediated metabolism of electrophilic or oxidant chemicals often used as antineoplastic agents, but also because of its apparent linkages to key signaling pathways known to be crucial for the survival, proliferation, and motility of malignant cells (8–16). In recent studies, we have shown that lack of RLIP76 in knockout mice leads to loss of nearly 4/5 of total GSH-conjugate (GSH-E) as well as anthracycline-transport activity, and widespread changes in GSH-linked antioxidant enzymes (17). In addition, endocytosis-mediated signal termination of varied signaling ligands including insulin, epidermal growth factor (EGF), and transforming growth factor (TGF) is affected by loss of RLIP76 (12, 18–21). RLIP76−/− mice develop a characteristic sensitivity to stress, particularly to ionizing radiation (17). These animal studies, as well as several studies by us and others in cell culture systems, have implicated RLIP76 as a part of stress defenses (22, 23), as well as a modulator of signaling through Ras, Bal (11), cdc-2 (19), cdc-42 (11), heat-shock proteins (24), EGF, TGF, and insulin (12, 18–21). Most remarkably, inhibition of RLIP76 transport function using antibodies to a cell surface epitope or depletion of RLIP76 using short interfering RNA (siRNA) uniformly causes apoptosis in a wide variety of histologic types of cancers in cell culture (25–31). The potential clinical applicability of RLIP76 inhibition or depletion depends on the demonstration of some degree of specificity of apoptosis directed at cancer cells, either due to greater expression of RLIP76 in cancer cells, greater dependence of cancer cell on RLIP76 to defend against stress, or a greater susceptibility of cancer cells to agents which deplete RLIP76.

In the present studies, we examined these possibilities by using anti-RLIP76 IgG as a specific inhibitor, as well as both siRNA and antisense DNA oligonucleotides for specific depletion of RLIP76 in cell culture models of malignant and nonmalignant cells and extended these studies in vivo, using a syngeneic B16 mouse melanoma model. Results presented in this article indicate that RLIP76 is expressed to a greater degree in malignant cells, that RLIP76 inhibition or depletion causes preferential toxicity towards malignant cells, and that anti-RLIP76 antibodies as well as RLIP76-depleting siRNA or antisense phosphorothioate oligonucleotides exert significant antineoplastic effects in an animal model of melanoma.
Materials and Methods

Reagents. Keratinocyte serum-free medium (K-SFM) supplemented with 5 ng/mL EGF, 50 µg/mL bovine pituitary extract, and 2 mM/L L-glutamine was purchased from Invitrogen, Carlsbad, CA. RPMI 1640, Ham's F12 K and DMEM medium, PBS, penicillin/streptomycin solution (P/S), fetal bovine serum (FBS), trypsin-EDTA, and trypan blue were purchased from Life Technologies, Inc., Grand Island, NY. Medium EGM-2 Bullet Kit was purchased from Cambrex BioScience (Walkersville, MD). Reagents for SDS-PAGE were purchased from Bio-Rad Laboratories (Hercules, CA). Doxoru- bincin (Adriamycin) was obtained from Adria Laboratories (Columbus, OH). [3H]Doxorubicin (specific activity, 57 mCi/ml) was purchased from Amersham Corporation (Arlington Heights, IL). [35S]PAP (specific activity, 3,000 Ci/mmol) was purchased from Perkin-Elmer Life and Analytical Sciences (Shelton, CT). FITC-conjugated goat anti-rabbit antibodies were purchased from Vector Laboratories, Inc., Burlingame, CA. Transmembrane Transfection Reagent kit was purchased from Qiagen (Valencia, CA). Ninety-six–well nitrocellulose membrane plates (pore size, 0.45 µm) used in transport studies were purchased from the Millipore Corp. (Bedford, MA). Fluorescin terminal deoxynucleotidyltransferase (TdT)–mediated nick end labeling (TUNEL) apoptosis assay kit was procured from Promega (Madison, WI). Sources of other reagents were the same as previously described (32–34).

Cell lines and cultures. Human small cell lung cancer (SCLC) lines H1618 and non–small cell lung cancer (NSCLC) lines H358 (brochioalveolar), PC-3 (human prostate), and B16 mouse skin melanoma were purchased from the American Type Culture Collection (Manassas, VA). Human lung bronchioepithelial cells (HLBEC) were kindly provided by Dr. John D. Minna, American Lung Cancer Research. Human small cell lung cancer (SCLC) lines H1618 and non–small cell lung cancer (NSCLC) lines H358 (brochioalveolar), PC-3 (human prostate), and B16 mouse skin melanoma were purchased from American Type Culture Collection (Manassas, VA). Human lung bronchioepithelial cells (HLBEC) were kindly provided by Dr. John D. Minna, American Lung Cancer Research. Human small cell lung cancer (SCLC) lines H1618 and non–small cell lung cancer (NSCLC) lines H358 (brochioalveolar), PC-3 (human prostate), and B16 mouse skin melanoma were purchased from American Type Culture Collection (Manassas, VA). Human lung bronchioepithelial cells (HLBEC) were kindly provided by Dr. John D. Minna, American Lung Cancer Research.

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520 nm, and red fluorescence at >620 nm. Photographs taken at identical exposure at 400 × magnification are presented. Apoptotic cells show green fluorescence and characteristic cell shrinkage.

Animal model. C57 BL/6 mice were obtained from Lexicon Genetics (The Woodlands, TX) and colonies were bred at the Animal Care Facility, University of Texas at Arlington, Arlington, TX. All animal experiments were carried out in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Twenty-one 16-week-old mice were divided into seven groups of three animals (treated with PBS, preimmune IgG, scrambled siRNA, scrambled phosphorothioate oligonucleotide, anti-RLIP76 IgG, RLIP76508-528 siRNA, and RLIP76508-528 phosphorothioate antisense oligonucleotide). All 21 animals were injected with 2 × 10^6 B16 mouse melanoma cell suspensions in 100 μL of PBS, s.c. Animals were examined daily for signs of tumor growth. Treatment was administered mouse melanoma cell suspensions in 100 μL of PBS, s.c. Animals were examined daily for signs of tumor growth. Treatment was administered when the tumor surface area exceeded 45 mm² (day 11). Treatment was administered mouse melanoma cell suspensions in 100 μL of PBS, s.c. Animals were examined daily for signs of tumor growth. Treatment was administered when the tumor surface area exceeded 45 mm² (day 11). Treatment was administered mouse melanoma cell suspensions in 100 μL of PBS, s.c. Animals were examined daily for signs of tumor growth.

Results and Discussion

Malignant cells contain a greater quantity of antigenically detectable RLIP76. Preliminary studies involving Western blot analyses of malignant cells indicated relatively larger amounts of RLIP76 in malignant versus normal cells. We therefore quantitated RLIP76 in various cell lines of different origin. Total RLIP76 was purified from the membrane fraction of several malignant cell lines, including human SCLC (H1618), NSCLC (H358), ovarian (OVAR-3), breast (MCF-7), prostate (PC-3), liver (HepG2), melanoma (DG-1), mouse melanoma (B16-F1), and nonmalignant human cell lines of endothelial (HUVEC, HLMVEC, epithelial (HLBEC), and aortic smooth muscle (HAVSMC) origin. Purification tables for each are provided (Supplemental Table A), and SDS-PAGE and Western blot analyses of purified protein against anti-RLIP76 IgG are shown (Supplemental Fig. A). Purification folds of 120 to 153 were observed, and single protein band of intact RLIP76 were seen in SDS-PAGE, which were recognized by the anti-RLIP76 antibodies in Western blot analyses. No significant contamination was observed. Purified protein was quantified by ELISA and results are presented (Table 1). Western blot analyses of crude membrane fraction from each cell with lanes loaded with equal amounts of crude protein (200 μg) are shown (Fig. 1). These results showed the presence of RLIP76 in all cell lines, and a relatively greater amount

Table 1. RLIP76 protein and transport activity in malignant and nonmalignant cell lines

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>RLIP76 Protein</th>
<th>Transport Activity (pmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg/10⁶ cells</td>
<td>Total crude protein (%)</td>
</tr>
<tr>
<td>Malignant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B16 (mouse melanoma)</td>
<td>71 ± 6</td>
<td>0.8</td>
</tr>
<tr>
<td>DG-1 (human melanoma)</td>
<td>63 ± 5</td>
<td>0.8</td>
</tr>
<tr>
<td>OVCAR-3 (human ovary)</td>
<td>53 ± 3</td>
<td>0.7</td>
</tr>
<tr>
<td>PC-3 (human prostate)</td>
<td>46 ± 3</td>
<td>0.6</td>
</tr>
<tr>
<td>H358 (human lung, NSCLC)</td>
<td>36 ± 3</td>
<td>0.6</td>
</tr>
<tr>
<td>H1618 (human lung, SCLC)</td>
<td>32 ± 3</td>
<td>0.5</td>
</tr>
<tr>
<td>MCF-7 (human breast)</td>
<td>15 ± 1</td>
<td>0.2</td>
</tr>
<tr>
<td>HepG2 (human liver)</td>
<td>17 ± 1</td>
<td>0.3</td>
</tr>
<tr>
<td>Nonmalignant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLMVEC (human lung endothelium)</td>
<td>19 ± 2</td>
<td>0.3</td>
</tr>
<tr>
<td>HLBEC (human lung epithelium)</td>
<td>22 ± 2</td>
<td>0.4</td>
</tr>
<tr>
<td>HAVSM (human aorta smooth muscle)</td>
<td>15 ± 1</td>
<td>0.3</td>
</tr>
<tr>
<td>HUVEC (human umbilical endothelial)</td>
<td>14 ± 1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

NOTE: Cell lines were cultured in respective medium as described in Materials and Methods, and homogenate was prepared from 1 × 10⁶ cells. RLIP76 was purified from total membrane fraction by DNP-SG affinity chromatography (28, 35), and quantified by ELISA. Purification table and SDS-PAGE of purified RLIP76 from different cell lines are presented in the Supplemental Data (Table A and Fig. A). Total membrane proteins were quantified by dye-binding method (39). For transport studies, plasma membrane fraction obtained from 2 × 10⁷ cells was enriched for IOVs by wheat germ agglutinin affinity chromatography (32). Transport activity was calculated from measurements of uptake of 14C-doxorubicin (specific activity, 8.2 × 10⁶ cpm/nmol) or 3H-DNP-SG (specific activity, 3.6 × 10⁶ cpm/nmol) into the IOVs in the absence or presence of 4 mmol/L ATP after 10 minutes of incubation at 37°C as previously described (32). Each transport study was done with three replicates in three independent experiments (n = 9).
of RLIP76 in malignant cells compared with nonmalignant cells. The exceptions to this were the MCF-7 breast cancer and the HepG2 hepatocellular carcinoma cell lines which contained very low levels of RLIP76, similar to that seen in nonmalignant cells.

Transport activity of RLIP76 is greater in malignant cells. Because RLIP76 represents the major transporter of doxorubicin as well as DNP-SG, as observed both in cell line studies (29) and in knockout mice (17), we examined whether the content of RLIP76

Figure 1. Comparison of RLIP76 levels in cultured malignant cells versus nonmalignant cells. Aliquots of crude detergent extracts of the membrane fractions of malignant cells (H1618, H358, OVCAR-3, PC-3, MCF-7, B16, HepG2, and DG-1) and nonmalignant cells (HAVSM, HUVEC, HLMVEC, and HLBEC), containing 200 μg protein were used for SDS-PAGE and Western blotting against anti-RLIP76 IgG as primary antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG as secondary antibody and developed with 4-chloro-1-napthol as chromogenic substrate. Results were quantified by scanning densitometry of the full-length RLIP76 protein band near 109 kDa. β-Actin was used as an internal control.

Figure 2. Comparison of cytotoxicity effects of anti-RLIP76 IgG and RLIP76 siRNA between malignant and nonmalignant cells. A, effect of preimmune IgG (gray columns) and anti-RLIP76 IgG (white columns; 37 μg/mL final concentration) on cell survival was determined by MTT assay (38). B, depletion of RLIP76 expression in cells by RLIP76 siRNA was done, using Transmessenger Transfection Reagent Kit, according to the manufacturer’s (Qiagen) instructions. Briefly, B16 melanoma (malignant) and HLMVEC (nonmalignant) cells were incubated for 3 hours with various concentrations of siRNA (ranging from 0 to 100 μg/mL final concentration) in Transmessenger Transfection Reagent, washed with PBS, followed by 48 hours of incubation at 37°C in medium before Western blotting with anti-RLIP76 IgG as primary antibody. C, the time-dependent effect of RLIP76 siRNA (fixed at 20 μg/mL final concentration) were also evaluated using eight malignant and four nonmalignant cells by determining RLIP76 protein levels by Western blot analyses at 0, 6, 12, 24, and 48 hours, after treatment of RLIP76 siRNA, using anti-RLIP76 IgG as primary antibody. D, MTT assay in eight malignant and four nonmalignant cells was also done 48 hours after treatment of siRNA: scrambled siRNA (gray columns) and RLIP76 siRNA (white columns; 20 μg/mL final concentration), using Transmessenger Transfection Reagent Kit (Qiagen). B and C, internal control (β-actin) is shown below the respective Western blots from each cell line. A and D, columns, mean from three separate determinations with eight replicates each; bars, ± SD (n = 24).
well as [3H-DNP-SG], using a standardized 96-well plate transport assay (32), revealed greater transport of both substrates in cells containing greater amounts of RLIP76 protein, and a general correlation between RLIP76 protein level and transport activity (Table 1). The greatest transport activity was found in melanoma cells, and transport activity in malignant cells was generally greater than in nonmalignant cells, with the exception of MCF-7 and HepG2. Lower expression of RLIP76 in MCF-7 and HepG2 correlated with lower transport rates in the crude membrane vesicles (Table 1). An excellent correlation was observed between measured total doxorubicin or DNP-SG transport rate and either RLIP76 protein (Table 1) or RLIP76 ATPase activity (Supplemental Table A).

RLIP76 inhibition or depletion caused preferential cytotoxicity in malignant cells. The effect of RLIP76 inhibition by anti-RLIP76 IgG, or depletion by RLIP76 siRNA508-528 were examined by incubation with varying concentrations of either anti-RLIP76 IgG or RLIP76 siRNA508-528 followed by MTT assay. The optimal timing for the appearance of apoptosis after exposure to anti-RLIP76 IgG has previously been determined to be 96 hours (26), and concentration dependence studies have shown saturable effects with maximum cell kill near 40 μg/mL. This previously observed behavior of anti-RLIP76 IgG was confirmed in present studies in which maximum inhibition was observed near 40 μg/mL by MTT assay (data not presented). Whereas the preimmune IgG caused no significant cell kill, anti-RLIP76 caused cell kill which was greater for the malignant cell lines (P < 0.01). The two malignant cell lines, MCF-7 and HepG2, which had the lowest expression of RLIP76 and lowest transport activity towards doxorubicin and DNP-SG were also least susceptible to anti-RLIP76 IgG (Table 1). Maximal susceptibility was observed with the melanoma cell lines and the SCLC cell line (Fig. 2A).

Studies of the concentration-dependent effects of RLIP76 siRNA508-528 revealed complete depletion of RLIP76 protein after 24 to 48 hours at a concentration of 20 μg/mL. The nonmalignant cells were somewhat less sensitive to RLIP76 depletion as compared with malignant cells. For example, 10 μg/mL siRNA affected B16 melanoma cells significantly more than HLMVEC (Fig. 2B). The time for maximal depletion of RLIP76 protein was also determined by exposing cells to 40 μg/mL RLIP76 siRNA508-528 and performing Western blot analyses after varying times of exposure. The relative resistance of nonmalignant cells to RLIP76 depletion by siRNA was also observed in these time-dependence studies (Fig. 2C), in which several nonmalignant cell lines (HLMVEC, HLBEC, HAVSM, and HUVEC), were relatively less affected with respect to RLIP76 protein at 24 and 48 hours as compared with the malignant cell lines (B16, DG1, Ovcar-3, PC-3, H358, and H1618). Again, the behavior of MCF-7 and HepG2 cell lines was more like the nonmalignant cells, with lower overall levels of RLIP76 expression, and lower sensitivity to depletion of RLIP76 by the siRNA. In MTT cytotoxicity assays, RLIP76 siRNA508-528 killed the malignant cells in a concentration-dependent manner with relative sparing of the nonmalignant cells, again with the exception of MCF-7 and HepG2 (Fig. 2D). The relative efficacy of cell kill was greater with the siRNA (Fig. 2D) as compared with anti-RLIP76 IgG (Fig. 2A) in these cell culture studies. TUNEL assay for apoptosis done with anti-RLIP76 IgG revealed results consistent with those observed with the MTT assay (Fig. 3), with greater apoptosis seen in the malignant as compared with nonmalignant cells.

Anti-RLIP76, siRNA or antisense DNA caused complete regression of B16 melanoma in mice. The above observations of the antineoplastic effects of RLIP76 depletion were examined in a syngeneic mouse B16 melanoma model. C57B mice were injected on their flanks with 1 × 106 B16-F1 melanoma cells and tumors were measured by calipers daily. When the surface area of the tumor (product of bidimensional measurements) exceeded 40 mm2 (day 11), animals were injected i.p. with 100 μL diluent alone (PBS), or the same volume of diluent containing 200 ng mL-1 of anti-RLIP76 IgG, RLIP76 siRNA508-528, or RLIP76 phosphorothioate antisense508-528.

Figure 3. Effect of anti-RLIP76 IgG on apoptosis as determined by TUNEL assay. Malignant cells (H358, OVCAR-3, PC-3, and B16 melanoma) and nonmalignant cells (HAVSMC, HUVEC) were treated with either preimmune serum or anti-RLIP76 IgG (37 μg/mL final concentration) for 24 hours and then washed off with PBS. Approximately 1 × 10⁶ cells were fixed onto poly-L-lysine-coated slides, and the TUNEL apoptosis assay was done using the Promega Apoptosis Detection Kit according to the protocol provided by the manufacturer. Slides were analyzed by laser scanning fluorescence microscope (Zeiss LSM510 META). Photographs taken at identical exposures (×400 magnification).

Apoptotic cells show green fluorescence.
Additional control animals were injected with preimmune IgG, scrambled siRNA, or antisense. Results of tumor measurements are presented with all control groups (including PBS, preimmune IgG, scrambled siRNA, or antisense) versus all treated groups (including anti-RLIP76 IgG, RLIP76 siRNA508-528, or RLIP76 phosphorothioate antisense508-528). Additional control animals were injected with preimmune IgG, scrambled siRNA, or antisense. Left, tumor measurements for the index lesion are presented with all control groups (●), preimmune IgG (●), scrambled siRNA (▲) or antisense (▼) versus all treated groups (*), anti-RLIP76 IgG (●), RLIP76 siRNA (+), or RLIP76 phosphorothioate antisense (○). Arrows, days on which treatment was given and repeated. Right, photographs of animals taken at 8 days after treatment are shown for all treatment groups.

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The present results provide strong evidence for the over-expression of RLIP76 in certain malignant cells as compared with nonmalignant cells. Greater RLIP76 expression corresponded to greater transport activity for doxorubicin as well as DNP-SG. Furthermore, cells with greater expression of RLIP76 were relatively more dependent on this stress-defense protein as compared with cells with lower expression. The specificity of therapy was evident from the lack of effect of preimmune IgG, scrambled siRNA, or antisense as compared with the PBS control. Results of recent studies investigating the efficacy of novel targeted agents for therapy of melanoma using a syngeneic mouse B16 melanoma model (40–44) were compared with the results of present studies. The present findings of regression of an established melanoma nodule with a single treatment 10 days after tumor implantation has not been observed in either of these previous studies in which partial treatment efficacy was observed only when treatment was begun at the time of tumor implantation.

Given the striking efficacy of this single-dose treatment in a highly resistant animal malignancy model as compared with previous therapies in the same model, advancing these therapies to a human clinical setting is strongly suggested. Animal studies are inherently limited because of the inability to obtain adequate toxicity data to compare the three different treatments with respect to relative toxicity. Because we did not explore the effects of varying doses in present studies, we cannot conclude which one of the three approaches would have greater clinical efficacy; this would of course depend on results of human clinical studies where therapeutic windows can be evaluated with respect to normal tissue versus cancer tissue toxicity. Whereas the anti-RLIP76 IgG was inferior to siRNA in cell culture studies, surprisingly, all three treatments were similar in efficacy on an equivalent dose basis in the animal model. The relatively greater efficacy of RLIP76 antibodies in vivo may be related to contributions of antibody-dependent cellular cytotoxicity, which is known to contribute significantly to the cytotoxic activity of other antibody therapies such as Rituxan and Herceptin (26). This would be a clear advantage of the antibody therapy. Because of the relatively controversial nature of gene therapy, the development of a humanized monoclonal antibody therapy targeting RLIP76 would seem to be a more reasonable avenue for further clinical development as compared with antisense therapy. However, the development of siRNA therapy would also seem to have one advantage: the relatively greater susceptibility towards RLIP76 depletion in malignant cells as compared with nonmalignant cells, an observation which could be exploited therapeutically.
Not all malignant cells overexpress RLIP76 as compared with nonmalignant cells: MCF-7 breast and HepG2 cells are clear examples. For MCF-7 cells, we have previously shown that RLIP76 represents only a 10% to 15% of total glutathione-conjugate and doxorubicin-transport mechanism, the remainder being accounted for by breast cancer resistance protein or multi-drug resistance protein-1 (45). These results do not rule out the possibility that other breast or hepatocellular carcinoma cell lines may be found which overexpress RLIP76. Taken together, our studies suggest that RLIP76, an important stress-defense signaling and transport protein, is present in relatively greater quantity in certain malignant cells including melanoma, ovarian cancer, prostate cancer, and lung cancer. Further studies in xenograft models with melanomas and the other susceptible cell lines are needed to show the general applicability of these observations prior to human clinical applications.

Acknowledgments


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Cancer Research

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