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Expression of lung resistance-related protein (LRP) in non-small cell lung carcinomas of smokers and non-smokers and its predictive value for doxorubicin resistance

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Immunohistochemical methods were used to determine the expression of the lung resistance-related protein (LRP) in 87 cases of untreated non-small cell lung carcinoma. LRP expression was detected in 39 patients (45%). A significant correlation between LRP expression and tumor resistance to doxorubicin was found (p=0.03, Fisher's exact test). An inter-relationship between LRP expression and gender, age, stage, lymph node status and survival times was not observed. Furthermore, a relationship between LRP expression and proliferation (cell cycle phases, take rate of lung tumors in nude mice and cyclin A expression) was not detectable. However, a correlation of borderline significance was noticed between LRP expression and the patients' smoking habits. Carcinomas in heavy smokers (more than 30 cigarettes daily) were more frequently LRP-positive than carcinomas found in non-smokers (p=0.09, Fisher's exact test).

Key words: Lung resistance-related protein, immunohistochemistry, non-small cell lung carcinomas, proliferation, smoking.

Introduction

Evidence increasingly indicates that several drug resistance mechanisms are present in non-small cell lung carcinomas. Both P-glycoprotein (P-gp) and glutathione S-transferase (GST)-π may play a role in drug resistance.[1,2] Recently, a possibly new membrane transport protein, termed lung resistance-related protein (LRP) because it was first identified in a multidrug-resistant lung cell line, has been isolated.[3] Since its identification, LRP has been found in a large number of resistant cell lines and tumors.[4–7] Because the cell lines were not drug selected, LRP may play an important role in inherent drug resistance. In a small group of patients with non-small cell lung carcinomas, LRP was present in most of the cases.[8] In contrast to studies conducted on leukemias[7] and advanced ovarian carcinomas,[9] the level of LRP expression in non-small cell lung carcinomas was not correlated with several clinical parameters. Therefore, our study determined the expression of LRP in a larger group of patients with non-small cell lung carcinomas (n=87), and analyzed the inter-relationships between LRP, tumor stage, metastasis, drug resistance *in vitro*, tumor cell proliferation, tumor take rate in nude mice and the patients' smoking habits.

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Materials and methods

Patients

Eighty-seven patients with previously untreated non-small cell lung carcinomas who had undergone surgery at the Chest Hospital Heidelberg were admitted into this investigation. The morphological classification of the carcinomas was conducted according to the WHO study.[10] All patients were staged at the time of their surgery according to the guidelines of the American Joint Committee on Cancer.[11] Of the 87 patients, 24 had stage I and stage II tumors and 63 had stage III tumors. The patients (11 women and 76 men) were 58 years old on average. Thirty-one patients did not exhibit any lymph node involvement while 56 patients did. Nineteen patients were non-smokers and 68 patients were smokers. All smokers were long-term smokers (longer than 10 years). The daily cigarette consumption rate among the smokers ranged from 1–10 cigarettes (nine patients), 11–20 cigarettes (22 patients), 21–30 cigarettes (12 patients), 31–40 cigarettes (13 patients) to more than 40 cigarettes (four patients). Cigarette consumption could not be precisely determined for eight smokers. None of the non-smokers were ex-smokers.

Immunohistochemistry

The biotin-streptavidin method was used to detect LRP and cyclin A.[1,12] Staining for LRP was carried out using the clone LRP-56 (monoclonal anti-human P110, dilution 1:20; Dunn Labortechnik, Asbach, Germany). A rabbit polyclonal antibody was used to measure cyclin A (cyclin A, H-432, dilution 1:50; Santa Cruz Biotechnology, Heidelberg, Germany). This antibody corresponds to amino acids 1–432 and represents the full length of human cyclin A. The antibody is specific for cyclin A and does not cross-react with other cyclins.

Alcohol-fixed and paraffin-embedded tumor sections were deparaffinized. After preincubation with hydrogen peroxide (0.3%), saponin (0.05%), unlabeled streptavidin and non-immunized normal serum, the primary antibodies were applied for 16 h at 4°C in a moist chamber. The most suitable concentration was determined in preliminary investigations. After a washing (three times) with phosphate-buffered saline, the sections were incubated for 45 min at room temperature in the presence of biotinylated goat anti-rabbit Ig or sheep anti-mouse Ig (1:50) as a secondary antibody (both with 5% normal human serum). Thereafter, the streptavidin biotinylated peroxidase complex (Amersham Braunschweig, Germany; dilution 1:100) was added. Following washing (three times) with buffered saline the peroxidase activity was visualized with 3-amino-9-ethylcarbazole. Counterstaining was performed with hematoxylin. Negative controls were carried out by omitting the primary antibodies and by substituting irrelevant antibodies for them. Positive controls were always present. The immunohistochemical staining was analyzed according to a scoring method which we have validated in a series of animal and human cell lines and human solid tumors. Staining was graded as either negative, weakly positive, moderately positive or strongly positive. The entire scoring process proceeded without any knowledge of the clinical parameters. Three observers independently evaluated the results from the immunohistochemical staining. The evaluations agreed in more than 90% of the samples. The other specimens (less than 10%) were re-evaluated and then given the classification most frequently assigned by the observers.

Detection of doxorubicin resistance *in vitro*

The short-term test for predicting resistance to doxorubicin has been previously described.[13] The basic principle involves measuring the changes in the incorporation of radioactive nucleic acid precursors into tumor cells after the addition of doxorubicin. Only fresh tumor specimens were processed. The biological variability within the individual samples was minimized by using large tumor sections.

Fat and necrotic areas were removed from the tumor tissue. Solid tumor material was first mechanically disrupted and filtered through gauze. Then the cells were sedimented and subsequently resuspended at a defined cell density (5×10^5 cells/ml^{-1}). Doxorubicin was tested over a concentration range encompassing four powers of 10. After incubation for 2 h, the radioactive nucleic precursor was added and the incubation was continued for an additional hour. Then 100 μl aliquots were pipetted from each test tube onto filter paper disks and dried in a stream of warm air. The non-incorporated radioactivity was extracted with ice-cold trichloroacetic acid. Filters were washed in ethanol–ether and the incorporated radioactivity was determined by liquid scintillation counting. Uptake values for the individual concentrations were expressed as percentages of the controls. Tumors were defined as being sensitive or resistant depending on prior clinical correlations.[13] The cut-off point chosen was 65% of the control value for a doxorubicin concentration of 10^{-2} mg/ml. After adding doxorubicin, the test results were assessed relative to the control samples.

Measurement of the tumor take in nude mice

BALB/c *nu/nu* mice (female, 6–8 weeks old) were maintained by conventional methods in macrolon cages at 27°C and 50% humidity. Autoclaved food and acidified water were provided as desired. The human tumor specimens were finely minced with scissors under aseptic conditions and suspended in a tissue culture medium (1 : 3 by volume). Then 0.3 ml of each suspension was injected s.c. into the flanks of three animals. If within a 3 month period growing nodules were detected and histologically confirmed, the tumor was assumed to have taken hold.

Cell cycle analysis

Flow cytometry analysis was carried out using an ICP-22 (Phywe, Göttingen, Germany). A mixture of propidium iodide (10 μg/ml) and 4′-6-diamidino-2-phenylindole (DAPI, 2 μg/ml of each in 0.15 M Tris-HCl buffer) was applied simultaneously with RNase

(1 mg/ml) after methanol fixation and protease digestion. For fluorescence excitation and detection we used UB1/BG 38 and FT 450/KV 540 filters (Schott, Mainz, Germany). Peripheral blood leukocytes from healthy donors were used as a calibration standard for DNA diploidy. Parallel measurements, both with and without the standard, were performed. Cell cycle analysis was conducted using integrated Gaussian fittings. A computerized subtraction of exponentially decreasing corrections beginning with the peak of cellular debris was included in the evaluation program. The cell cycle analysis was omitted in cases that showed interspersed cell populations (about 30%).

Statistical analysis

The correlations between the different parameters were statistically evaluated by using Fisher's exact test. Life table analysis according to Kaplan and Meier was performed for the overall survival rate. Different groups were compared by the log rank test. A p value of less than 0.05 was considered to be significant.

Results

The expression of LRP in the untreated non-small cell lung carcinomas of 87 patients was determined by immunohistochemistry. Figure 1 shows a typical expression pattern of LRP. LRP-56 staining was cytoplasmic and vesicular in our study. LRP expression was detected in 39 cases (45%). We compared the expression of LRP with the response of the carcinomas to doxorubicin *in vitro* (incorporation of radioactive nucleic acid precursors as a percentage of the controls). The test threshold between sensitive and resistant tumors was based on earlier clinical correlations between test results obtained with the short-term test and the response *in vivo*.[13,14] In the present investigation, 24 carcinomas (28%) were classified as sensitive and 63 carcinomas (72%) as resistant. Figure 2 shows the expression of LRP (negative, low, moderate and high staining) in both resistant and sensitive non-small cell lung carcinomas. It appears that LRP-positive carcinomas are more frequently resistant than LRP-negative ones. In order to evaluate whether the expression of LRP does indeed correlate with tumor resistance to doxorubicin, we used Fisher's exact test to analyze the data (Table 1) and did in fact discover a significant correlation between these two parameters (p=0.03, Fisher's exact test). A significant relationship between LRP expression and gender, age, stage or lymph node status was not observed (Table 2). Furthermore, a relationship between LRP expression and several proliferation markers (cyclin A expression, S, G_2 and M phases and tumor take rate in nude mice) was not found. A

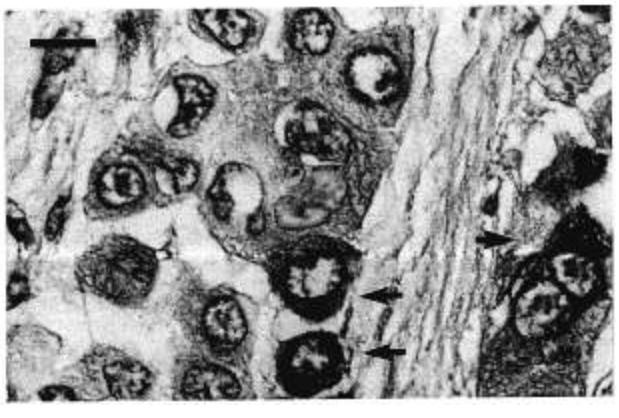

Figure 1. Immunohistochemical staining of LRP in lung cancer. Arrows: positive immunostaining. Bar: 20 μm.

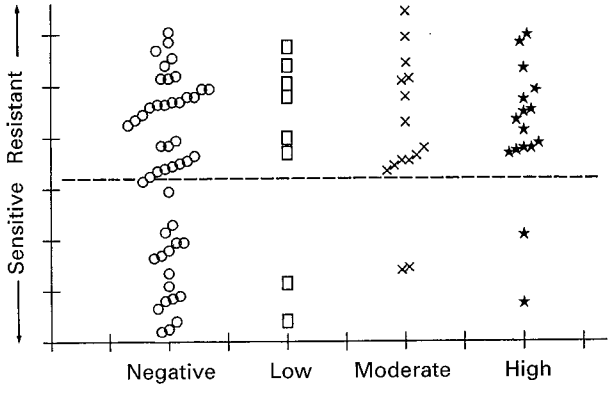

Figure 2. LRP expression in non-small cell lung carcinomas according to the result of the short-term test. Abscissa: LRP expression (negative, low, moderate and high staining). Ordinate: incorporation of nucleic acid precursors in tumor cells after addition of doxorubicin as a percentage of the controls. Tumors were defined as being sensitive or resistant depending on prior clinical correlations (cut-off point: 65%).

Table 1. Expression of LRP in doxorubicin-sensitive and doxorubicin-resistant non-small cell lung carcinomas (n=87)

	LRP-negative	LRP-positive (%)	Fisher's exact test
Sensitive	18	6 (25)	
Resistant	30	33 (52)	p=0.03

relationship to the patient survival rate was also not noticed (Table 3).

In contrast, a significant relationship between the smoking habits of the patients and LRP expression in tumors was detected (Table 4). Carcinomas in heavy smokers (more than 30 cigarettes daily) were more frequently LRP positive (11 of 17 cases) than carcinomas in non-smokers (six of 19 cases). This result is of borderline statistical significance (p=0.09, Fisher's exact test).

Table 2. Expression of LRP in non-small cell lung carcinomas subdivided according to clinical parameters

	LRP-negative	LRP-positive (%)
Gender		
female	5	6 (55)
male	43	33 (43)
Age		
<58 years	21	22 (51)
>58 years	27	17 (39)
Stage		
I,II	14	10 (42)
III	34	29 (46)
Lymph node status		
negative	19	12 (39)
positive	29	27 (48)

Table 3. Relationship between LRP expression and proliferation markers in non-small cell lung carcinomas

	n	LRP-negative	LRP-positive
Cyclin A			
negative	72	10	8
positive		27	27
S, G_2 and M phases (%)			
≤22	50	13	14
>22		14	9
Take rate			
no	67	19	14
yes		14	20
Median survival (weeks)	87	76	60

Table 4. Distribution of LRP-negative and LRP-positive tumors of smokers (n=68)[a] and non-smokers (n=19)

Cigarettes (daily)	LRP-negative	LRP-positive (%)
0	13	6 (32)
1–30	25	18 (42)
>30	6	11 (65)

[a]In eight cases cigarettes consumption could not be determined.

Discussion

LRP has been found in varying levels in different tumors. Izquierdo *et al.*[4] analyzed the expression of LRP in a panel of 61 human cancer cell lines derived from cancers of the colon, kidney, ovary, breast and brain, as well as from melanoma and leukemia. The expression of P-gp and multidrug resistance-related protein (MDR) was also investigated. LRP and MRP were expressed in 78 and 87% of the tumor cell lines, respectively. P-gp was detected at relatively low levels in only 24% of the samples. An examination of 174 tumor specimens that comprised 27 tumor types yielded an LRP expression in 63% of the cases. Izquierdo *et al.*[9] analyzed 57 stage III and IV ovarian carcinomas for LRP and for P-gp. Sixteen percent of the cases showed positive immunostaining for P-gp and 77% for LRP. Positive immunostaining of LRP was not associated with any other examined prognostic factor. However, positive LRP immunostaining in treated advanced ovarian carcinomas appears to indicate a poor response to treatment. List *et al.*[7] examined bone marrow specimens from 87 patients with acute myeloid leukemia for an expression of LRP. The overexpression of LRP was associated with an inferior response to induction therapy. This agrees with the data which we obtained for childhood acute lymphoblastic leukemia (unpublished data). In our present analysis of non-small cell lung carcinomas, we found LRP expression in 45% of the cases and a significant relationship to *in vitro* resistance to doxorubicin. The *in vitro* short-term test that is used to determine resistance is able to predict resistant tumors with a high degree of accuracy.[13–15] In a cooperative study, results from the short-term test were compared with results from chemotherapy and it was determined that in 96% of the cases tumors that were resistant in the test were also clinically progressive.[13]

Presently, various reports suggest that cell populations that exist in human lung tumors have several resistance mechanisms operating at once.[1,2] This indicates that each lung tumor has its own unique resistance factor profile. This investigation discovered an inter-relationship between LRP, GST-π and P-gp. Thirty-two percent of the non-small cell lung carcinomas simultaneously expressed LRP and P-gp, while 41% of the carcinomas expressed LRP and GST-π (data not shown). In contrast, other resistance-related factors that were examined earlier (topoisomerase II, metallothionein and heat shock proteins) did not exhibit a significant correlation with LRP.

Since the growth rate of tumors is an important determinant of tumor response to chemotherapy, we also investigated the inter-relationship between LRP

expression and several proliferation markers. We determined the proportion of cells in S, G_2 and M phases by flow cytometry, the human tumor take rate in nude mice, and the expression of cyclin A. Cyclins are regulatory proteins for cyclin-dependent kinases that are synthesized and degraded by various means at specific points during the cell cycle. Cyclin A achieves its peak during S phase and closely correlates with the proportion of S phase cells.[16] In the present study, we found no correlation between cell proliferation and LRP expression.

It is well known that exposing cells to chemical carcinogens results in a resistant phenotype. Thorgeirsson *et al.*[17] and Fairchild *et al.*[18] reported that MDR gene transcripts are elevated in both preneoplastic and neoplastic nodules found in the rat liver during carcinogenesis. We also used a model for chemical carcinogenesis in which only one carcinogen, *N*-nitrosomorpholine, was administered and examined whether overexpression of P-gp occurs in hepatocellular carcinomas. As our experiments demonstrate, the overexpression of P-gp is readily apparent even after the carcinogen has been withdrawn.[19] Furthermore, we found that non-small cell lung carcinomas that affect smokers are more frequently resistant and express a greater quantity of P-gp and GST-π than do the corresponding tumors in non-smokers.[20] The present investigation shows that a relationship between smoking habits and LRP expression exists. Lung carcinomas in heavy smokers were more frequently LRP-positive, but this result was only of borderline significance. Dingemans *et al.*[8] studied 39 normal lung tissues for LRP expression and related the data obtained to the number of pack-years smoked. They discovered that LRP intensity levels were not correlated to the number of pack-years smoked, although a trend was noted for the higher LRP intensity levels in patients who smoked for more than 10 pack years.

In conclusion, we found a significant correlation between LRP expression and resistance to doxorubicin in non-small cell lung carcinomas. We demonstrated that a relationship of borderline significance exists between the patients' smoking habits and LRP expression. A significant relationship between gender, age, stage, lymph node status and LRP expression was not observed. Furthermore, a correlation between LRP and several proliferation markers could not be found.

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