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REVIEW ARTICLE

Early Diagnosis of Lung Cancer by Detection of Tumor Liberated Protein

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Tumor liberated protein (TLP) is a protein that can be used to reveal the early development of a tumor. Besides being formed in the tumor, TLP is released in the blood when a patient starts producing cancer cells, which in turn enables the physician to intervene at a stage when the cancer is operable. To date, the available studies of tumor markers in lung cancer patients are CEA, NSE, TPA, Chromogranine, CA125, CA19-9, and Cyfra 21-1. The sensitivity and specificity for serum markers ranges between 50 and 90%, depending on the study and the clinical samples analyzed. Most of these markers show an increased rate of positivity as the stage advances. There are very limited data on TLP to draw any firm conclusion regarding the diagnostic value of this marker. TLP has been detected in 53.1% of non-small cell lung cancer (NSCLC) patients (N = 534) with 75% being positive in the early stage (stage I) and dropping to 45% in the late stage (stage IV). However, 7.6% blood donor sera and 17.4% chronic lung disease sera have also tested positive. In a confirmation study, the specificity was 89.94% and the sensibility was 63.63% from stage III to IV NSCLC patients. In an initial study of TLP as a marker for early detection in stage I, NSCLC patients showed a sensitivity of 66.7% and a specificity of 80% for TLP compared to a sensitivity of 33.3% for CA19-9, 11.1% for Cyfra 21-1 and CA125, and 0% for CEA; the specificity for all four of the latter markers was 100%. Using immunohistochemical analysis with peroxidase anti-peroxidase (PAP), we observed that NSCLC cells were positive; we used the specific rabbit antiserum to TLP, which turned out negative in the presence of 1 mg/ml of the synthetized peptide. The pre-serum was also negative. The same reactivity was found early in the modified epithelial cells of interstitial lung fibrosis and might be a predictive marker of cell transformation. The site of the peroxidase positivity was cytoplasmic, of diffuse and/or granular type. J. Cell. Physiol. 203: 1–5, 2005. © 2004 Wiley-Liss, Inc.

EARLY DETECTION AND RESEARCH STRATEGY IN LUNG CANCER

Lung cancer is the main cause of mortality in the world for men between 35 and 70 years of age. The highest incidence rates are found in Europe and North America. It is estimated that there were approximately 375,000 cases of lung cancer in Europe in 2000, 303,000 in men and 72,000 in women; the resulting deaths totalled about 347,000 (280,000 men and 67,000 women) (Tyczynski et al., 2003). It is clear that lung cancer is a very important health problem for men, considering the mortality rate associated with the disease and the fact that the choices of therapy for this tumor are limited. Air pollution, polynuclear aromatic hydrocarbons mostly derived from car traffic, radon and tobacco smoking surely play a fundamental role in causing this disease and therefore the first approach must be prevention measures. However, even though tobacco smoking and environmental factors are certain causes of risk, they alone do not seem sufficient to justify the differences found in mortality and incidence for lung cancer within a country and between various countries. One must consider other important risk factors that act as independent factors or as modifiers or modulators of the effects of smoking. For this reason, new approaches are required to counteract this particular epidemic.

The discovery of biomarkers, that is markers of the biological process associated with cell growth, represents a basic target in tumor diagnosis, since they can be used for early diagnosis, tumor relapse and metastasis, as well as for prognosis, site of tumor, and monitoring of therapy.

Several studies have shown that the expression of different biomarkers is frequent in cell lines of lung cancer, and many authors have considered the presence of these markers in blood as an index of the extension of disease, prognosis, and response to therapy. Some tumor markers (NSE, Chromogranine, CEA, TPA, and CYFRA 21-1) have been evaluated in many patients with small cell lung cancer (SCLC) as well as with NSCLC. However, the diagnosis and follow-up of this pathology are not yet satisfactory, although a relationship with clinical relevance has been found for the NSE and chromogranine markers in SCLC; the CYFRA 21-1 marker, on the other hand, has been useful only in the follow-up of NSCLC for the squamous subtype (Soichiro et al., 2001). Recently, there has been renewed interest in screening as spiral computerized tomography can detect small asymptomatic lesions more effectively than conventional radiography. Although cure rates for such lesions are very good, there is to date no evidence for the effectiveness of mass screening strategies (Tyczynski et al., 2003).

TUMOR ASSOCIATED ANTIGENS IN HUMAN LUNG CANCER

In 1983 a tumor associated antigen (TAA) was isolated from non small cell lung (NSCL) cancer (Tarro et al., 1983) and named tumor liberated protein (TLP) (Tarro, 2000). TLP is composed of soluble lipoglycoproteins; its size measures between 48.1 and 61 Å and it has an isoelectric point less than 7.0 and a molecular weight of 214,000 Da. It was first analyzed for amino acid composition and then some partial sequences were

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obtained. A 100 kDa (CSH-275) fragment was identified in TLP and one epitope was used as an antigen to produce antibodies directed towards specific sites of TLP by inoculation in rabbits (Tarro et al., 1993). This antiserum was named CSH-419 and used by some authors for research on TAA in lung and colorectal cancer patients (Garaci et al., 1996; Guadagni et al., 2000). A reasonably high level of purification of TLP enabled us to obtain an initial amino acid sequence which in turn led to the synthesis of the following three specific peptides from lung and colon cancer (Tarro, 1999): ArgThrAsnLysGluAlaSerIle; GlySerAlaXpheThrAsn; AsnGlnArgAsnArgAsp. A fourth peptide, GlyProProGluValGlnAsnAlaAsn, was obtained from TLP which was purified from an urogenital tumor.

Recent studies with rabbit antibody raised against a peptide from lung and colon cancer, made up of eight amino acids, revealed that TLP is produced in the cytoplasm of the malignant cells (Table 1, Fig. 1A). Since it was not detected in normal tissues, we concluded that it was a tumor-specific antigen (Tarro et al., 1998). Our work on TLP indicates that this protein appears to be a new tumor marker with potential clinical applications. Using Western blot analysis, we were able to show that TLP was expressed in all lung cancer-derived cell lines, in breast cancer-derived cell lines, and in colorectal-derived cell lines that were studied (Tarro et al., 1998). Since lung and intestinal tissues have a common embryonic origin, it is not unreasonable to suggest that TLP might be tumor-specific antigen for at least some of the malignancies that arise from tissues of epithelial origin. Since we were not able to detect TLP in normal lung tissues, nor in the osteosarcoma cell line SAOS-2 (Tarro et al., 1998), the notion that TLP is indeed a tumor-specific antigen is supported. On the basis of the immunohistochemical studies which revealed a large amount of TLP in the columnar epithelial cells and lumen (secreted TLP), as illustrated in Figure 1A, it appears that TLP is a cytoplasmic antigen which is specific for epithelial cell-derived neoplasms. Preincubation of the antibody with the corresponding immunizing antigenblocked the immunocytochemical reaction, thus confirming the specificity of the antibody (Tarro et al., 1998).

Immunohistochemical evaluation of the role of TLP

Immunohistochemically, all the lung cancer specimens with high or medium levels of differentiation showed TLP cytoplasmic immunostaining (Table 1). The evaluated colorectal carcinomas also were positive for TLP expression (Tarro et al., 1999). No TLP protein was detected in the surrounding normal tissues or in inflammatory cells. In some specimens this specific staining was also recognized, as well as in the lumen of atypical glands and in bronchial secretion. This last observation, taken together with TLP expression in all the adenocarcinoma-derived cell lines (including MCF-7 and HT-29) and tumor specimens, could suggest that TLP is a secretory product of the neoplastic cells. On the other hand, the lower differentiated squamous carcinomas, the small cell lung carcinomas (well known to be an aggressive type of lung cancer) and the two sarcomas did not exhibit any TLP immunostaining (Tarro et al., 1999).

Combining the immunohistochemical data with the results of the Western blot analyses, we conclude that TLP is a cytoplasmatic antigen specific for epithelial cell-derived neoplasms only, because it was not detected in non-neoplastic tissues cells, in neuroendocrine tumors, or in mesenchymal cell-derived tumors and normal cells (Rasi et al., 2000). In addition, finding that TLP is detectable in neoplasms and tumor cell lines with common embryological features seems to suggest that TLP might be an "oncofetal antigen."

IMMUNOISTOCHEMICAL TEST BY PEROXIDASE ANTI-PEROXIDASE (PAP)

Immunoreactivity for TLP was found in neoplastic tissues by anti NQRNRD serum prepared after rabbit immunization obtained by using the sequence length of six amino acids (Tarro, 1999) according to the pattern already performed in the Cold Springs Harbor Laboratory (Tarro et al., 1993).

The immunostaining was always cytoplasmic, with a low-to-absent background. All adenocarcinomas showed a high expression level of TLP (Fig. 1C,D). The protein was distributed either in small granules or in larger accumulations (Fig. 2A,B); in some specimens, TLP was detected in the lumen of atypical glands and in the bronchial secretions (Fig. 2C,D), suggesting that TLP could be considered a secretory product of the neoplastic cells. We found cytoplasmic-specific TLP staining of various expression levels in all medium- and well-differentiated specimens of squamous carcinomas (Fig. 1B). However, TLP was not detected in the infiltrating lobular carcinoma of the breast (Fig. 3C), urothelial carcinoma (Fig. 3D), or in the myocardium

TABLE 1. Reactivity of rabbit antiserum to the TLP-derived peptide (RTNKEASIC)

Organ	Origin of tissue	Peroxidase positivity
1. Lung	Normal adult	0
2. Lung	Fetus 18 weeks	0
3. Lung	Interstitial inflammation	0
4. Lung	Bronchiolo-alveolar adenocarcinoma	3+
5. Lung	Acinar adenocarcinoma	3+
6. Lung	Papillary adenocarcinoma	3+
7. Lung	Epidermoid carcinoma (differentiated)	2+
8. Lung	Epidermoid carcinoma (non-keratinizing)	0
9. Lung	Small cell carcinoma	0
10. Lung	Sarcoma	0
11. Lung	Malignant mesothelioma	0
12. Skin	Malignant melanoma	0
13. Breast	Infiltrating duct carcinoma	0
14. Breast	Intraductal adenocarcinoma	0
15. Thymus	Malignant thymoma	0
16. Endometrium	Adenocarcinoma	0
17. Bladder	Papillary urothelial carcinoma	0
18. Colon	Adenocarcinoma	2+

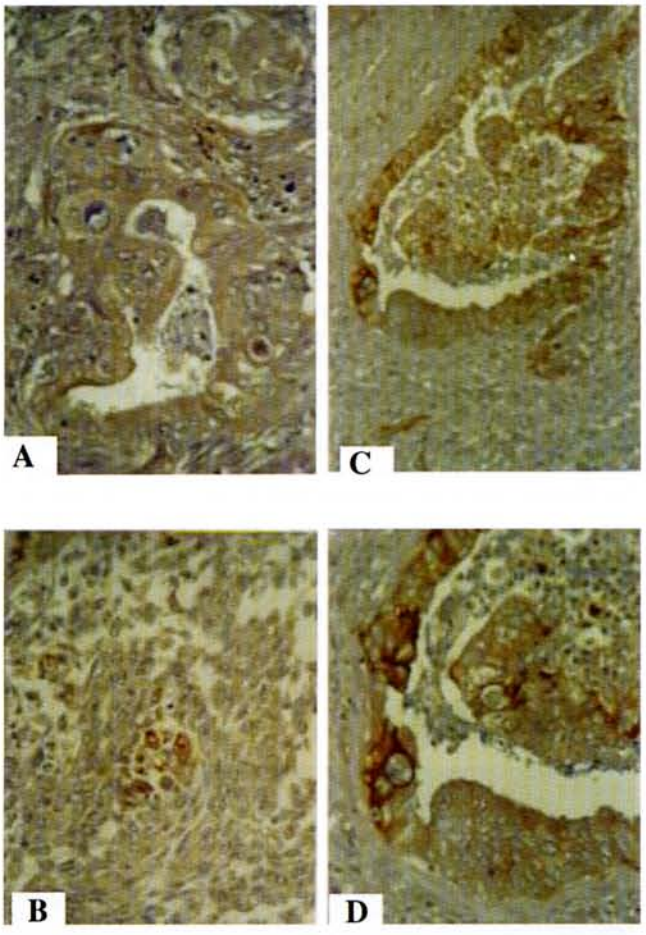

Fig. 1. Histological section of a lung adenocarcinoma incubated with the rabbit antiserum to the tumor liberated protein (TLP) peptide RTNKEASI (**A**) and of a squamous lung carcinoma (**B**) or of a lung adenocarcinoma (**C** and **D**) incubated with the rabbit antiserum to the TLP peptide NQRNRD. The brown staining represents the distribution of TLP in these malignant tissues (A, B and D: ×480; C: ×320).

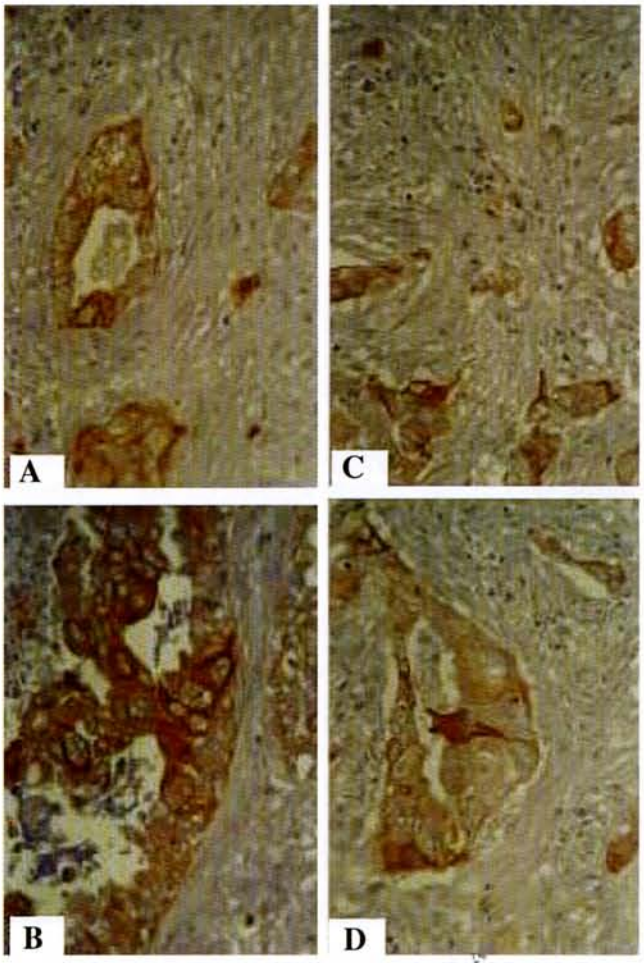

Fig. 2. TLP immunohistochemical expression by the antibody to NQRNRD sequence. A representative staining in a lung adenocarcinoma (**A**: ×320; **B**: ×480; **C**: ×320; **D**: ×480).

(Table 2). A mild expression level of TLP was also detected in the colorectal adenocarcinoma examined (Fig. 3A). Some authors detected TLP in 53.1% of NSCL cancer patients, with 0% positivity in patients with tumors other than NSCL cancers, 7.6% positivity in unknown blood donors, and 17.4% positivity in patients with chronic lung diseases having an elevated risk for lung cancer (Garaci et al., 1996). The antibody to NQRNRD sequence stains the alveolar epithelium modified in interstitial lung fibrosis (Fig. 3B). This epithelium changes from flat to cubical; this alteration is termed cubical allomorphism, indicating a return of the alveolar epithelium to the look of the fetal lung before exhibiting respiratory functions. Lung parenchyma with diffuse interstitial fibrosis does not display any respiratory function due to the complete vanishing of septal capillary circulation in this pathology, as in the fetal lung (Perna et al., 1999). The parenchyma with such modifications is more exposed to neoplastic transformation (Garaci et al., 1996). The study of the distribution of anti-TLP antibodies in healthy people and patients with neoplasia, or in other patients affected by lung inflammatory pathologies who could fall under the category of lung neoplasia, is very interesting (Esposito et al., 1997). In fact, the reactivity signal in general pathologies is a useful test to assess the specificity and positive predictive value of the assay. Finally, we believe that sensitivity can increase sensibility, with improvement of the Ist generation test (Tarro and Esposito, 2002).

POLYCLONAL ANTIBODY AND "ELISA SANDWICH"

Previous studies have shown the reactivity of the rabbit antiserum obtained against the peptide sequence RTNKEASI (Tarro et al., 1993, 1998, 2001; Garaci et al., 1996; Tarro, 1999, 2000, 2002; Rasi et al., 2000).

Since this sequence is analogous to that present in corin (RTQKEASI), a proteic component of human myocardium, polyclonal antibodies were produced by PRIMM (Milan, Italy) in rabbits against both variants 1 and 2 of corin w.t., for the sequences of junction or deletion, respectively, to determine whether the TLP was homologous or only analogous to the corin.

An ELISA sandwich was prepared using the rabbit antiserum anti-RTNKEASI-KLH as a capturing antibody, purified for immune affinity (IA) with peptide, whereas different antisera were used as the tracing antibody: poly Ab anti var 1 deletion—BSA purified with protein A; poly anti var 1 deletion—KLH purified for IA with peptide; poly anti var 1 junction—BSA purified with protein A; poly anti var 1 junction—KLH purified for IA with peptide; poly anti var 2 deletion—BSA purified with protein A; poly anti var 2 deletion—KLH

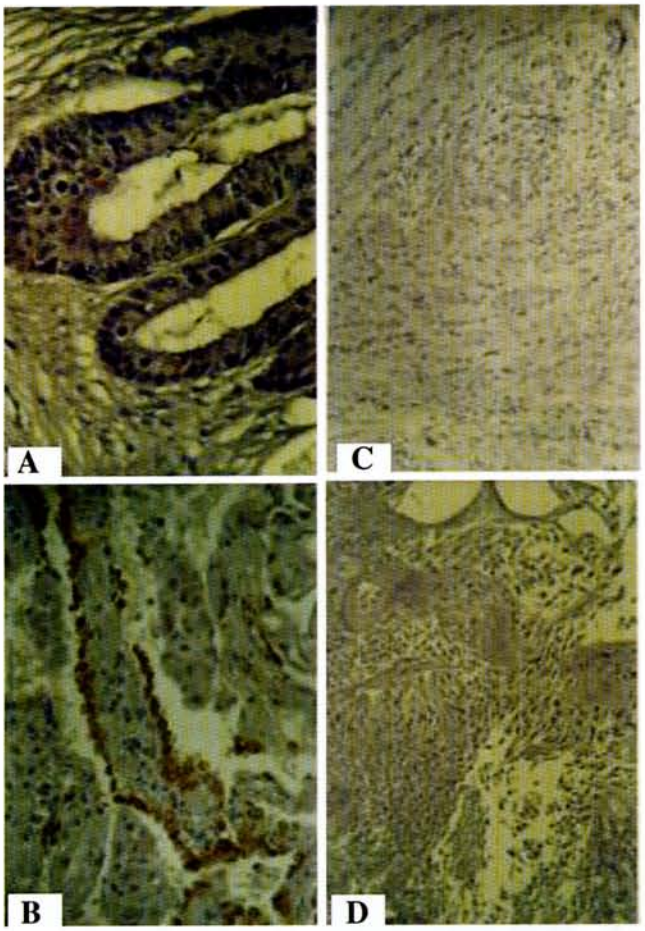

Fig. 3. TLP immunohistochemical expression by the antibody to NQRNRD sequence. A representative staining in a colo-rectal carcinoma (**A**: ×480). The antibody to NQRNRD sequence stains the alveolar epithelium modified in the interstitial lung fibrosis (**B**: ×480). Negative for TLP immunostaining an adenocarcinoma of the breast (**C**: ×480) and an urotelial carcinoma (**D**: ×320).

purified for IA with peptide; poly anti var 2 junction—BSA purified with protein A; poly anti var 2 junction—KLH purified for IA with peptide.

All antibodies were purified by the pool of antisera of two rabbits immunized and conjugated with biotine or with horseradish peroxidase (HRP). Antigen samples were human myocardium and normal lung. The concentration of HeLa cells and NSCLC extracts were 1 and 5 μg/ml, respectively. As the control, an ELISA standard plate was prepared to check the correct procedure for the use of the tested antibodies (gel filtration, lyphilization, and dissolution in H_2O, conjugation); the coating was performed with the antigens by which the antibodies against the corin variants were induced in rabbits. The

TABLE 2. Reactivity of rabbit antiserum to the TLP-derived peptide (NQRNRD)

Organ	Origin of tissue	Peroxidase positivity
1. Lung	Normal adult	0
2. Lung	Interstitial fibrosis	+
3. Lung	Adenocarcinoma	3+
4. Lung	Epidermoid carcinoma	2+
5. Heart	Myocardium	0
6. Breast	Infiltrating duct carcinoma	0
7. Colon	Adenocarcinoma	+
8. Bladder	Urothelial carcinoma	0

sera were first purified against peptide + BSA and then after gel filtration on a NAP 25 column (Pharmacia, Uppsala, Sweden), as required by the conjugation protocol ($NaHCO_3$ 0.1 M + 0.9% NaCl). After gel filtration, lyophilization, and dissolution, the purified sera had higher antibody titers. The serum + EZ link was tested at different dilutions (1:10, 1:50, 1:100, 1:1,000) and a significant loss of activity was measured. When we tested the sera at lower dilutions (1:10, 1:50, 1:100), the results confirmed the loss of activity.

The resuspension of lyophilized serum in water does not make it possible to obtain a homogeneous solution, but rather one containing precipitates. Therefore, the conjugation on 0.3 mg in 50 μl of total and non-specific IgG, conducted according to the protocol, might have limitations for the final yield. Furthermore, we evaluated the immunohistochemical expression of TLP using the polyclonal antibodies to corin w.t. variants 1 and 2 for the sequence of junction and deletion. TLP was not detected in the differentiated adenocarcinomas by all the rabbit antisera (Fig. 4A,B), whereas the immunostaining was low to absent in the myocardium (Fig. 4C,D).

AIMS AND PERSPECTIVES FOR TLP

Ongoing studies have attempted to achieve the complete sequence of TLP (Tarro, 2002), which in turn

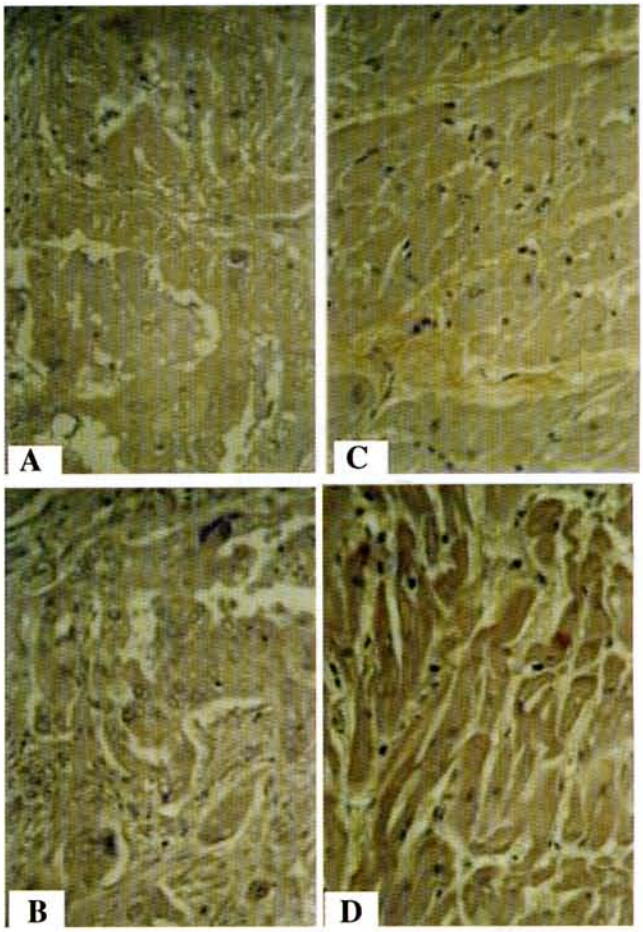

Fig. 4. A lung adenocarcinoma negative for TLP immunostaining by the antibody to the corin variant 1 of junction (**A**: ×480) and by the antibody to the corin variant 2 of junction (**B**: ×480). A human myocardium negative for immunostaining by the antibody to the corin variant 1 of junction (**C**: ×480) and slightly positive for immunostaining by the antibody to the corin variant 2 of junction (**D**: ×480).

should enable in vitro preparations of large quantities of TLP by genetic engineering. The RT-PCR of the TLP fragment (300 bp), from the A549 cell line, was subcloned into the PGEM-T easy plasmid (vector) (Promega, Madison, WI), resulting in the T-easy TLP construct (3,300 bp). The fragment (300 bp) could be cleaved with EcoRI. A putative open reading frame was deduced (Tarro, 2002).

The availability of such antigen preparations will facilitate future studies on the role of TLP in human malignancy. It could also enable the preparation of an assay for early diagnosis of the corresponding tumors (Soichiro et al., 2001; Tarro and Esposito, 2002) and might even be useful in the generation of a specific anticancer vaccine to prevent neoplastic disease in subjects at risk of developing cancer, through the stimulation of the immune system by preparing on attack against cells that express this protein.

With the aim of identifying with the potential role of TLP in early lung cancer detection, anti-TLP antibodies were studied in lung cancer patients (Tarro and Esposito, 2002). Low sensitivity could result from the appearance of specific immunocomplexes able to block the detection of TLP, when anti-TLP antibodies appear in the serum of the patient. In view of this possibility, we performed a study to determine the presence of anti-TLP antibodies in sera from NSCL cancer patients and also to determine whether these antibodies represent an immune response to TLP related to human lung cancer (Esposito et al., 1997). It was also important to show the correlation of the antibody with the time of appearance of the TLP in the blood of the patients (Tarro and Esposito, 2002). Furthermore, in 1997 it was shown for the first time that this antigen obtained from human lung cancer is capable of producing a humoral immune response (Esposito et al., 1997).

Hybridomas producing anti-TLP monoclonal antibodies (MAbs) were produced using the somatic fusion method with spleens of mice immunized with TLP-derived peptide RTNKEASIC (paapep), conjugated with KLH and BSA (Tarro et al., 2001). Among the MAbs selected, eight belonged to the IgM class and two to the IgG class. One of these MAbs, 1F2/11, was selected for the setting up of an ELISA for titration of TLP in biological fluids. The test is based on the competition of the MAbs for antigen coated on microtiter plates and antigen in solution. The ELISA is able to recognize not only pep9aa but, more importantly, a crude preparation of TLP, TLP in tumor extracts and TLP in human serum (Tarro et al., 2001).

For only six lung adenocarcinomas (Perna et al., 2001), we used the monoclonal antibody (MAb) to TLP diluted 1:100 on a single section with the goal of looking for a possible relationship between intracellular TLP concentration, degree of neoangiogenesis, and extension of angioinvasion (Ray and Stettler-Stevenson, 1994; Rak et al., 1995; Fontanini et al., 1997; Perna et al., 1999). In two cases, we observed a high level of intracellular TLP, a moderate degree of neoangiogenesis and lack of angioinvasion; in four cases, a high level of intracellular TLP corresponded with extended neoangiogenesis and clear-cut angioinvasion (Perna et al., 2001).

In conclusion, the results of our study emphasize the importance of neoangiogenesis and angioinvasion in the evolution of malignancies capable of strongly influencing survival, as well as the antigenic proteic products associated with neoplastic tissue (TLP among them) and their possible role in the process of metastasis.

New studies presented in this review article show that a rabbit antibody raised against a second peptide of six amino acids (NQRNRD) from NSCLC belonging to the same protein previously detected and characterized (TLP) is produced in the cytoplasm of malignant cells. Our study indicates that this protein appears early in the modified epithelial cells of interstitial lung fibrosis and might be a predictive marker of cell transformation. These findings might allow for the preparation of an ELISA with a first sandwich between the polyclonal antibody against RTNKEASI and the serum sample capturing the antigen, and then a second immunological reaction between the second rabbit antibody (anti-NQRNRD) and the previous complex for revealing the reactivity. A specificity >95% and an increased sensitivity would yield a good method for the detection of TLP antigen in the early stages of lung cancer.

LITERATURE CITED

Esposito C, Tarro G, Cuomo N, Morelli F. 1997. Anti-TLP antibodies in lung cancer patients. Int Med 5:191–194.

Fontanini G, Lucchi M, Vignanti S, Mussi A, Ciardiello F, De Laurentis M, De Placido S, Basolo F, Angeletti CA, Bevilacqua G. 1997. Angiogenesis as a prognostic indicator of survival in non-small cell lung carcinoma: A prospective study. J Natl Cancer Inst 89:881–886.

Garaci E, Sinibaldi P, Rasi G. 1996. A new tumor-associated-antigen of non-small cell lung cancer: Tumor liberated protein (TLP) a possible tumor marker. Anticancer Res 16:2253–2256.

Guadagni F, Graziano P, Roselli M, Mariotti S, Bernard P, Sinibalsi-Vallebona P, Rasi G, Garaci E. 2000. Differential expression of a new tumor-associated-antigen TLP during human colorectal cancer tumorigenesis. Am J Pathol 154:993–999.

Perna A, Tarro G, Esposito C, Baldi C, Annunziata S, Morelli F, Cuomo N, Di Spirito A. 1999. Neoangiogenesis and prognosis in lung cancer. Int Med 7:111–113.

Perna A, Tarro G, Perna F, Esposito C. 2001. Immunochemical evaluation of endothelal damage by angioinvasivity. Int J Clin Invest 9:31–33.

Rak JW, St Croix BD, Kerbel RS. 1995. Consequences of angiogenesis for tumor progression, metastasis and cancer therapy. Anticancer Drugs 6:3–18.

Rasi G, Sinibaldi-Vallebona P, Serafino A, Bernard P, Pierimarchi P, Guarino E, Faticanti-Scucchi L, Graziano P, Guadagni F, Garaci E. 2000. A new human tumor-associated antigen (TLP) is naturally espressed in rat DHD-K12 colorectal tumor cells. Int J Cancer 85:540–544.

Ray JM, Stettler-Stevenson WG. 1994. The role of matrix metalloproteases and their inhibitors in tumor invasion, metastatis and angiogenesis. Eur Resp J 7: 2062–2072.

Soichiro A, Hideki K, Neomichi I, Yasushi N, Masayuki S, Michiko A, Takayuki K. 2001. Optimal combination of seven tumor markers in prediction of advanced stage at first examination of patients with non-small cell lung cancer. Anticancer Res 21:3085–3092.

Tarro G. 1999. Tumor liberated protein (TLP). Its potential for diagnosis and therapy. Anticancer Res 19:1755–1758.

Tarro G. 2000. An overview of the lung tumor liberated protein (TLP). Characterization of the genetic immunologic profile. Int Med 8:5–8.

Tarro G. 2002. Characterization of a fragment containing a putative TLP cDNA sequence. Anticancer Res 22:2693–2696.

Tarro G, Esposito C. 2002. Progress and new hope in the fight against cancer: Novel developments in early detection of lung cancer. Int Med 10:7–11.

Tarro G, Pederzini A, Flaminio G, Maturo S. 1983. Human tumor antigens inducing in vivo delayed hypersensitivity and in vitro mitogenic activity. Oncology 40:248–254.

Tarro G, Marshak DR, Perna A, Esposito C. 1993. Antigenic regions of tumor liberated protein complexes and antibodies against the same. In: Carpi A, Sagripanti A, Grassi B, editors. Third Internation Congress. Advances in Management of Malignancies. Pisa, Italy: 6/10 December 1993. Biomed & Pharmacother 47:237.

Tarro G, Giordano A, Esposito C, Perna A, Claudio PP. 1998. Immunoistochemical characterization of tumor liberated particles (TLP) expression pattern in lung cancer. Anticancer Res 18:2365–2370.

Tarro G, Perna A, Esposito C, Morelli F, Cuomo N, Di Spirito A. 1999. Aims and perspectives for tumor liberated protein (TLP). Riv It Ric Med Chir 7:43–46.

Tarro G, Esposito C, Perna A, Cuomo N, Di Spirito A, Morelli F, Perna F, Maio A, Nolli ML. 2001. Production and characterization of anti-TLP derived peptide (RTNKEASIC) monoclonal antibodies. Int Med 9:55–61.

Tyczynski JE, Bray F, Parkin DM. 2003. Lung cancer in Europe in 2000: Epidemiology, prevention, and early detection. Lancet Oncol 4:45–55.