RhoA protein expression in primary breast cancers and matched lymphocytes is associated with progression of the disease

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Abstract. RhoA protein is over-expressed in breast cancer and other solid tumors and has been used in tumor biopsies as a quantitative tumor marker for progression, stage and prognosis in molecular detection strategies. Measuring protein markers in plasma or blood cells is preferred to tumor biopsies as it represents a minimally invasive, repeatable measurement that can be followed over time. In this study we evaluated the hypothesis that quantitative RhoA protein expression in circulatory lymphocytes is identically associated with the same tumor clinico-pathological features found in biopsies. RhoA protein levels were analyzed by Western blotting in circulating lymphocytes isolated from 52 consecutive patients with breast cancer and in 34 paired breast tumor biopsies from the same case study, and compared with the following clinico-pathological features of the patients: histological grade, tumor size, steroid receptor status, lymphonode status, proliferative activity and prognosis [Nottingham Prognostic Index (NPI)]. We observed that the level of circulatory, peripheral lymphocyte RhoA expression reflected that found in the matched biopsy of the same patient. Furthermore, similarly to previous reports regarding breast cancer tissue biopsies, the level of RhoA protein expression in both biopsies and in circulatory lymphocytes was positively associated with tumor size, grade, proliferative activity of the tumor biopsy and NPI, while there was no significant association of RhoA protein expression with either estrogen- or progesterone-receptor expression. Our study demonstrated that the association of lymphocyte RhoA protein expression with classical clinico-pathological parameters closely corresponded with that observed for RhoA protein expression in the tumor biopsies. We propose that measurement of RhoA expression in the circulatory lymphocytes of breast cancer patients can be used to predict breast cancer occurrence, progression and prognosis and may prove valuable in the management of cancer patients.

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Introduction

A main area of research in the development of modern tumor markers is the study of the physio-pathological role of signal transmission molecules in cancer development and progression. In this context, the use of Rho-GTPases has been extensively studied and characterized in tumors. The Rho-GTPase subfamily consists of small, 20 to 30-kD monomeric GTP-binding proteins that are able to bind GDP/GTP and hydrolyze GTP leading to the activation of downstream effector molecules and subsequent cellular response (1). Multiple cell-cycle components are impacted by Rho proteins and over-expression of the RhoA isoform can induce DNA synthesis either independently of, or in cooperation with, Ras (2). RhoA is also required for both endothelial cells and migrating tumor cells to cross the vascular endothelium in order to enter either the blood or the lymphatic vasculature and metastasize to distant sites (3-6). The activation of RhoA has induced prostate, pancreatic, breast cancer invasion (7-10) and we recently demonstrated that invasion in metastatic human breast cells is coordinated by a sequential RhoA-dependent signalling pathway (11). It is now clear that RhoA may be associated with the carcinogenesis, progression, neoangiogenesis and metastasis of human breast tumors.

This contribution of Rho proteins to cancer cell proliferation, survival, invasion and metastasis has stimulated the study of the expression pattern of RhoA in many types of human cancers. Tumor over-expression of RhoA has been reported in ovarian (12), bladder (13), hepatocellular (14-15), lung (16), liver (17), colon (18) and testicular cancers (19-20), where it is associated with a later tumor stage (21). In particular, in breast cancer tumor, RhoA over-expression has been found to correlate with a more advanced neoplastic stage and was associated with a shorter disease-free and overall survival (22-24), suggesting that it could be an independent prognostic marker. These data demonstrate that RhoA is a marker of clinical relevance in breast cancer patients. However, its potential clinical relevance is largely limited by the fact that RhoA needs to be measured in primary tumor tissue that is not always available and, furthermore, is not useful for the frequent monitoring of the clinical course of the disease.

Circulating biomarkers are easily measurable and amenable to repeated analysis as is generally required for monitoring the clinical stage of the disease, prognosis, response to therapies, and toxicities (25-27). Sources for circulating biomarkers include plasma proteins and protein expression in the immune cell component. Although blood may not be the primary target tissue in disease, there is evidence that many diseases are associated with an inflammatory component and may be evaluated and monitored in peripheral blood lymphocytes (28-31). Indeed, the association between immune cells and cancer has been known for over a century (32-37). Each stage of cancer development is susceptible to regulation by immune cells that form the first line of immune defence and regulate the activation of adaptive immune responses, releasing soluble and bioactive mediators that induce the mobilization and infiltration of additional inflammatory cells into damaged tissue. During this process, adaptative immune cells, such as lymphocytes, distinguish themselves from innate leucocytes by the expression of somatically generated, diverse antigen-specific receptors exerting multiple effector functions that are continually fine-tuned as tissue microenvironments are altered (38).

Hypothesizing that breast cancer cells elicit a specific T-cell response associated with and dependent upon the characteristics of the solid tumor, we quantitatively evaluated RhoA expression in circulatory peripheral lymphocytes and matched tissue biopsies from patients with diagnosed breast cancer in relation to the main clinico-pathological characteristics of the disease to determine its utility as a progression and/or prognostic tumor marker in molecular detection strategies. We found that the level of circulatory lymphocyte RhoA expression strongly reflected that found in the matched biopsy of the same patient and that, indeed, the expression levels of RhoA protein in circulating lymphocytes were associated with the same patient clinico-pathological characteristics as those from the tumor biopsy. The present study verifies previous observations in tumor biopsies suggesting that RhoA plays an important role in tumor progression and demonstrates that RhoA protein levels in circulating lymphocytes could be considered for a potential, non-invasive diagnostic assay for breast cancer, and therefore needs to be validated in a larger population.

Materials and methods

Patients. Blood samples (n=32) and breast cancer surgical specimens (n=14) were obtained from a consecutive series of patients with a first diagnosis of primary breast cancer histologically confirmed at the Women's Department of the National Cancer Institute in Bari, Italy. In a further consecutive series, blood and tissue samples were collected from the same patients (n=20). Before undergoing routine surgery, all patients signed an informed consent form authorizing the institute to utilize their removed biological tissues for research purposes. Routine staging procedures were adopted for the determination of stage disease extension according to UICC criteria (39). The patients underwent surgery before receiving any therapy. Just after the surgical removal of biological tissues, the pathologist selected samples intended for routine diagnostic practice and for research activities from the primary tumor and from contiguous macroscopically non-involved breast tissues. The characteristics of these tissue samples were successively confirmed by H&E histological analysis. The cytohistological tumor differentiation grade was determined as previously reported (40). The Nottingham Prognostic Index (NPI), combining tumor size, lymph node stage and histological grade information, was utilized to score each patient as follows: patients with NPI values <2.5 were associated with an expected best prognosis, those with an NPI of 2.5-3.5 with an expected intermediate prognosis and an NPI >4.5 was associated with the poorest prognosis (41). Hormone receptor expression was determined by immunohistochemical assays and the cases were categorized as positive or negative according to the cut-off value of 10% of positive immunostained cells (42). Tumor proliferative activity was determined as the percentage of tumor cells expressing the growth-related MIB antigen by immunohistochemical assay (42).

Separation of peripheral circulatory lymphocytes. Peripheral blood lymphocytes were isolated from whole blood by Ficoll-Hypaque™ centrifugation. Diluted anti-coagulated blood was layered over Ficoll-Hypaque and centrifuged (30 min at 1800 rpm) over a step gradient consisting of a mixture of the carbohydrate polymer Ficoll™ and the dense iodine-containing compound metrizamide. Red blood cells, polymorphonuclear leukocytes and granulocytes were centrifuged through the Ficoll-Hypaque while mononuclear cells consisting of lymphocytes together with some monocytes were banded over it and recovered at the interface. Lymphocytes were then purified by washing the sample with PBS and centrifuging it 2 times at 1200 rpm for 10 min.

Sample preparation and Western blotting. For the analysis of protein expression, lymphocytes and tissues were first homogenized in a homogenization buffer (HEPES 5mM, EDTA 0.5 mM, pH 7.2 with KOH) to which 2 μl of protease inhibitor cocktail per ml of buffer, 1mM DTT, 100 mM PMSF and 0.1% NP40 were added. An aliquot of 30 μg of total protein was than heated at 100˚C in SDS sample buffer (6.25 mM Tris-HCl, pH 6.8, containing 10% glycerol, 3mM SDS, 1% 2-mercaptoethanol and 0.75 mM of bromophenol blue) and separated by 10% SDS-polyacrylamide gel

electrophoresis. The separated proteins were transferred onto Immobilon-P (Millipore) for immunoblotting. The membrane was first washed for 30 min with TBS containing 0.1% Tween-20 and was then blocked with 5% dried fat-free milk in TBS for 1 h followed by washing twice with TBS. The blocked membrane was incubated with the primary antibody overnight at 4˚C followed by three washes with TBS. RhoA-protein expression was analyzed using an overnight incubation with a RhoA-specific goat polyclonal antibody (1:1000, Santa Cruz Labs, CA). The membrane was incubated with rabbit anti-goat IgG (1:2000, Cell Signaling, MA) for 1h, washed 3 times with TBS and subsequently developed with ECL (Amersham Biosciences).

RhoA protein expression was quantified by densitometry in which the blot film was scanned with an imaging scanner (Epson, Milan, Italy) and analyzed with NIH ImageJ imaging software. After Western blot analysis, proteins bound to the membrane were stained with Coomassie blue to confirm that identical amounts of protein had been transferred. To quantify protein levels, the relative RhoA O.D. values of the lymphocytes were related by densitometric analysis to that of the breast cancer cell line, MCF7, which was set as 1.

Tissue fractionation. Tissue specimens were first homogenized in ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and homogenated by five passes through a 20-gauge needle to obtain the total homogenate. An aliquot was removed for the determination of total cellular protein. The total lysate was centrifuged at 100,000 x g for 1 h, in order to obtain a membrane pellet and a cytosol supernatant. Fifty micrograms of each of the separated cellular fractions were extracted in SDS sample buffer and were analyzed by Western blotting.

Immunohistochemistry (IHC). Immunohistofluorescence studies were performed on formalin-fixed tissue sections embedded in paraffin wax. The breast cancer tissues, obtained from patient breast biopsy specimens, were fixed in 20% neutral buffered formalin for 24 h and embedded in paraffin. Three-micron serial sections were obtained from tissue blocks, deparaffinized with xylene and rehydrated in an ethanol series. For antigen retrieval, the sections were microwaved at 500 W for 10 min in citrate buffer (pH 6) and endogenous peroxidase activity was blocked with 30 ml/l hydrogen peroxide solution.

Slides were incubated with RhoA-specific goat polyclonal antibody (1:100, Santa Cruz Labs) overnight at 4˚C in a humidified chamber. The bound antibody was visualized using a biotinylated secondary antibody, avidin-biotin peroxidase complex, and 3-amino-9-ethylcarbazole (Ultra Vision Detection System anti-Polyvalent, HRP/DAB, Lab Vision Corporation). For negative control sections, primary antibody was replaced with phosphate-buffered saline and processed in the same manner. Images were obtained on a Leica optical microscope and analysed with QWin Leica software.

Statistical analysis. The Kruskal-Wallis non-parametric ANOVA test was applied to analyze RhoA expression between the different grades and NPI stages while the Mann-Whitney non-parametric test was applied to normal and tumor tissues and lymphocytes, age, menopausal status, size, node status and the positive vs. negative classes of estrogen receptor (ER), progesterone receptor (PgR) and proliferative activity (MIB). In each case, the negative classes were defined as tumors having values <20 in IHC analysis. Correlation analysis of RhoA expression with ER, PgR and MIB expression was performed with the Spearman's rank non-parametric test. All comparisons were performed with InStat (GraphPad Software). For all analyses $P<0.05$ was considered significant.

Results

Expression of RhoA protein in breast tumors and non-involved tissues. Tissue lysates were prepared from 34 samples of human breast tumor tissue (T) and the contiguous, non-involved breast tissues (NT) from the same patients, and subjected to immunoblot analysis using anti-RhoA polyclonal antibody. Fig. 1A shows representative immunoblot analysis results for breast tumor and adjacent, non-tumor tissues. A specific band representing the 21-kD RhoA protein was detectable in both the tumor and non-tumor specimens of all patients. We found RhoA to be over-expressed in 100% of the human tumor breast samples analyzed (0.41±0.15 vs. 2.08±0.44 in contiguous non-involved and tumor tissues, respectively, $P=0.0003$, $n=34$, ratio RhoA/actin O.D. values normalized to MCF-7 expression).

Fig. 1B shows immunohistochemistry of RhoA in a series of images from 3-μm sections of contiguous, non-involved tissue (a) at the edge of and from a pT1N1M0 (G3) mammary tumor (b) and a magnified view of the tumor tissue (c). These images verify that RhoA expression was higher in the tumor lobules (T) than in normal lobules (N) of the contiguous peritumoral breast tissues. Furthermore, the magnified view of the tumor shows that RhoA expression was mostly cytosolic, with increasing staining in the membrane of the tumor tissue. As membrane bound RhoA is considered to be the active, GTP-bound form (43), this suggests that not only is RhoA over-expressed in the tumor cells but it is also more active.

To further address the question of whether RhoA proteins are more activated in breast cancer, we then compared the amount of the membrane-associated RhoA (active, GTP bound) with the amount in the cytosol (inactive, GDP bound) in a series of breast tumors and their corresponding non-tumor tissue, using Western blot analysis. As can be seen in Fig. 1C, in addition to the increased RhoA expression in the cytosol fraction of tumor tissue (T), there was an increased expression of the membrane-associated RhoA protein in the tumor tissue compared to non-tumor (N) tissue, further suggesting that there is also increased RhoA activity in breast cancer.

Comparison of tissue and lymphocyte RhoA expression and their association with tumor clinico-pathological characteristics. Peripheral blood lymphocytes were separated and analysed for their RhoA protein expression in Western blots using the anti-RhoA polyclonal antibody. In part of the above case study, RhoA protein expression in patient tumor biopsies and their matched isolated, peripheral blood

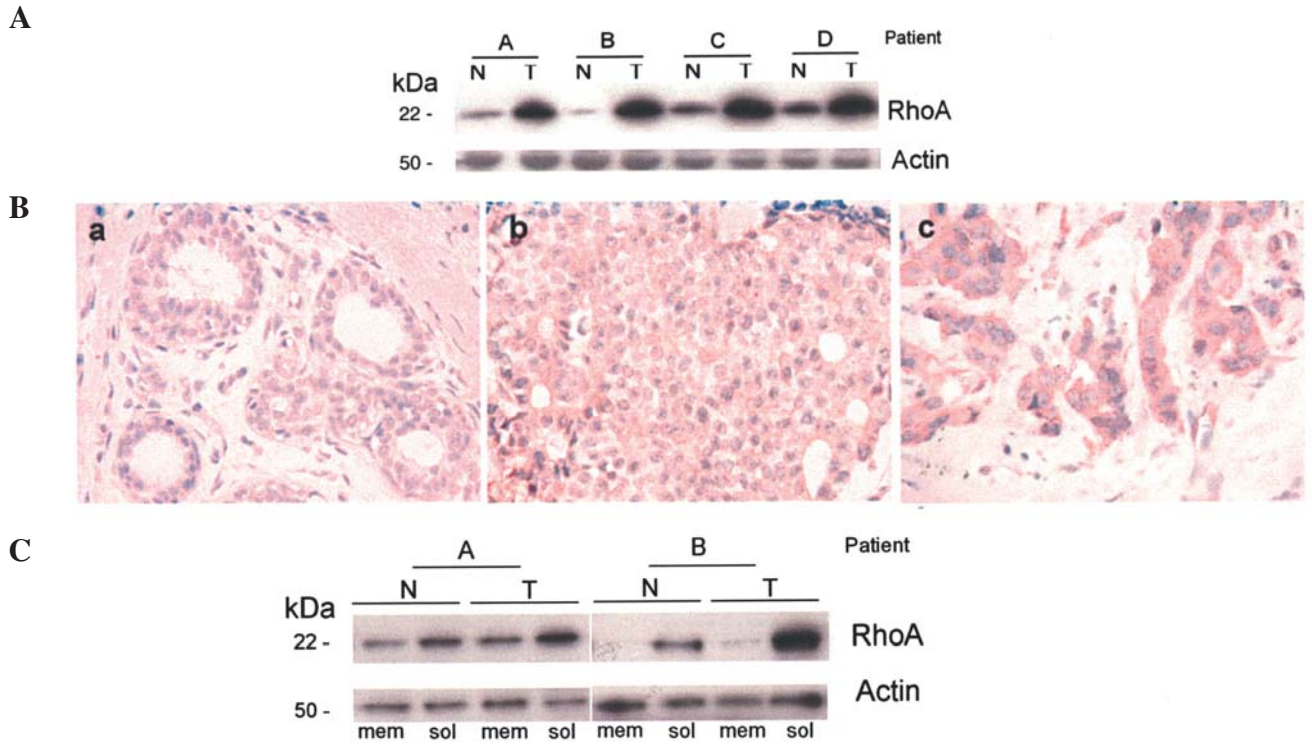

Figure 1. Characterization of RhoA expression in tumor and non-tumor tissue. (A) Representative immunoblotting analysis of RhoA protein expression levels in breast tumor (T) and in its contiguous, non-tumor breast tissue (N) for four patients. (B) Representative immunohistochemistry for RhoA protein expression in an infiltrated ductal carcinoma from a pT1N1M0 (G3) mammary tumor (b), from contiguous, non-involved tissue (a) and a magnified view of the tumor tissue (c). (C) Representative immunoblotting analysis of the subcellular distribution of membrane-associated RhoA (mem) and cytosol RhoA expression (sol) in breast tumor and the corresponding non-tumor tissue of two patients.

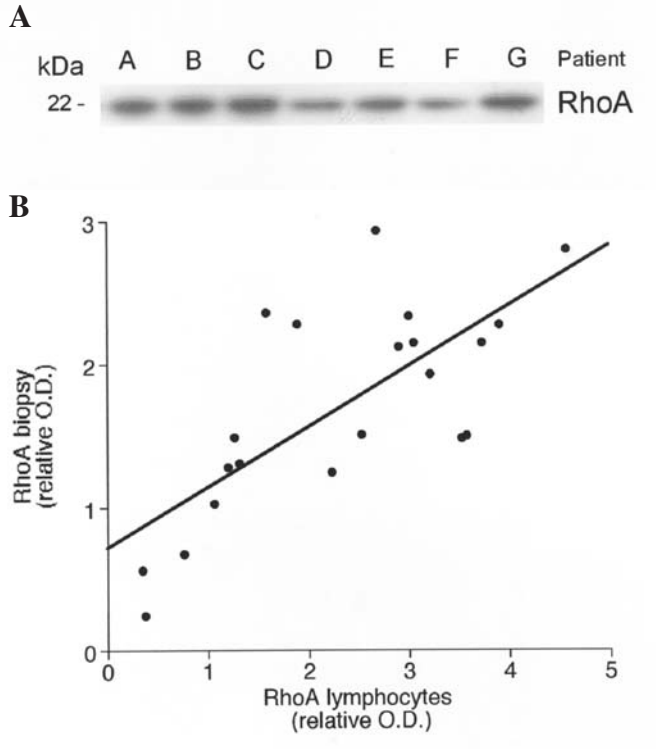

Figure 2. Characterization of RhoA expression in lymphocytes. (A) Representative immunoblotting analysis of RhoA expression levels in circulatory lymphocytes from patients with breast cancer. Lymphocytes were extracted for total protein and RhoA expression analyzed by Western blot analysis as described in Materials and methods. (B) Spearman's rank correlation relationship between lymphocyte RhoA protein expression and tumor RhoA protein expression in the same patients.

lymphocytes were analysed by Western blotting. Fig. 2A illustrates a typical Western blot of lymphocytes using the anti-RhoA polyclonal antibody, and Fig. 2B is a summary of the quantification of the Western blot analysis of lymphocytes vs. biopsies. As can be seen in Fig. 2B, we observed that the level of RhoA expression in circulatory lymphocytes closely reflected the level found in the matched biopsy of the same patient (r^2=0.548, p=0.0145, n=20). These data support the hypothesis that, at least for RhoA, the circulatory lymphocytes closely mirror the expression that the protein has in tumor tissue.

However, this tight correlation does not determine whether the RhoA expression levels in lymphocytes are associated with the same clinical characteristics as the RhoA levels in the tissue samples. Therefore, the RhoA protein expression was then analyzed with respect to the main, clinico-pathological disease parameters for both a series of tissue biopsies and peripheral lymphocytes (Table I). When analyzed according to the tumor cytohistological differentiation grade, both tissues and circulatory lymphocytes from patients with grade 3 tumors (poorly differentiated) expressed significantly more RhoA than did tissues from patients with grade 1 tumors (well-differentiated) (P<0.01). Tissue and circulatory lymphocyte RhoA expression was also positively associated with tumor size and, while not quite significant, both tissue and lymphocyte RhoA expression tended to increase with the presence of regional nodal metastases. To relate the biomarker level of RhoA expression with a whole prognosis indicator for our operable breast cancer patient series, we analyzed the relationship of RhoA expression with respect to

Table I. RhoA expression in lymphocytes (n=52) and tissue (n=34) of breast cancer subjects.

	Lymphocyte			Tissue		
	Media ± SE	Median	P	Media ± SE	Median	P
Age (years)						
≤54	1.99±0.23	1.76		1.90±0.34	1.68	
>54	2.51±0.24	2.73	0.01	1.90±0.14	2.02	ns
Menopausal status						
Pre	2.11±0.26	1.99		1.83±0.29	1.50	
Post	2.37±0.22	2.14	ns	1.98±0.15	2.14	ns
Tumor size (cm)						
2	1.84±0.19	1.49		0.51±0.17	0.16	
2	2.59±0.30	2.52	0.06	1.17±0.22	1.00	0.0700
Node status						
Negative	1.91±0.19	1.67		0.67±0.15	0.38	
Positive	2.52±0.26	2.18	ns	1.27±0.31	0.80	ns
Grade						
1	1.95±0.24	1.99		0.74±0.35	0.58	
2	1.87±0.26	1.57		0.52±0.17	0.16	
3	2.97±0.36	3.31	0.01	1.57±0.26	1.52	0.0100
NPI						
<2.5	1.85±0.20	1.58		0.48±0.20	0.16	
<3.5	2.64±0.28	2.37		0.95±0.28	0.78	
<4.5	2.94±0.25	3.15	0.02	1.82±0.25	1.67	0.0034

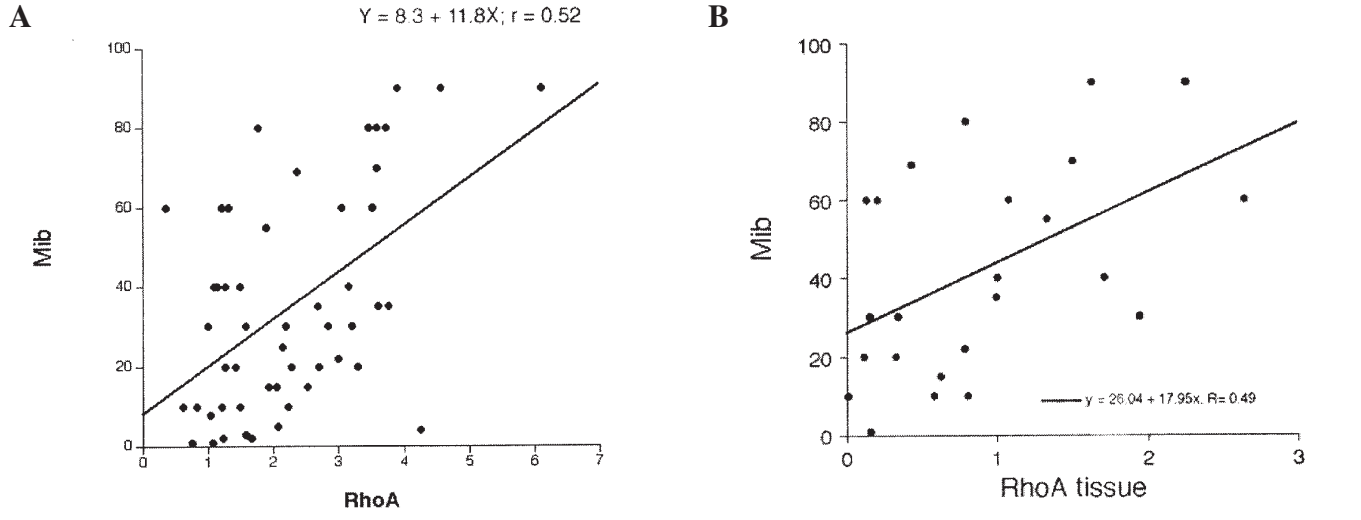

Figure 3. Spearman's rank correlation relationship between the percentage of cells immunostained for proliferative marker, MIB, and (A) lymphocyte RhoA protein expression and (B) tissue RhoA protein expression as described in Materials and methods.

the Nottingham Prognostic Index (NPI) (44). This index combines tumor size, lymph node stage and histological grade and the scoring system in which the group with NPI values <2.5 had the best prognosis, an NPI of 2.5-3.5 indicated an intermediate prognosis and NPI >4.5 was associated with a poor prognosis, has been validated in prospective studies (45-46). As seen in Table I, both tissue and circulatory lymphocyte RhoA expression significantly increased with increasing NPI and patients with NPI >4.5 (worst prognostic group) had significantly increased tissue and lymphocyte RhoA expression when compared with patients from both the 2.5-3.5 NPI group and the <2.5 NPI group (P=0.0034 vs. P=0.014, in tissue vs. lymphocytes, respectively). There was no significant correlation between RhoA levels and either estrogen- or progesterone-receptor expression levels in either tissue biopsies or in circulatory lymphocytes.

Lastly, linear regression analysis of the samples revealed a high positive correlation between the percentage of immunostained cells with the proliferative marker, MIB, and both tumor (Fig. 3A) and circulatory lymphocyte (Fig. 3B) RhoA expression (r=0.49, P=0.0260 vs. r=0.580, P=0.0074 in tissue and circulatory lymphocytes, respectively). This positive association of biopsy RhoA with MIB has been previously reported for breast tumors (47).

Discussion

In this study we demonstrated for the first time that lymphocyte RhoA expression levels directly reflect the RhoA expression levels observed in biopsies from the same patient. We found that tissue and lymphocyte RhoA protein expression was significantly higher in higher-grade tumors compared to lower-grade tumors, and in tumors with a poorer prognosis compared to those with a better prognosis. Our results confirmed the evidence for the implication of a small GDP/GTP binding protein in immunologic events associated with neoplastic diseases, but most notably, this is the first report of the relationship between RhoA expression in lymphocytes and clinico-pathologic factors such as histologic grade and tumor dimension in biopsies from the same patients and the first report of a protein that is identically expressed in peripheral blood lymphocytes and biopsies of patients with breast cancer, which may provide important clues regarding the pathogenesis of this disease.

Data from a number of laboratories have clearly shown that increased RhoA expression in the primary tumor is of clinical significance and that increased expression indicates a more aggressive phenotype and poor prognosis (14-16,18,20-24,47). Fritz *et al* (48) addressed the question of a putative relevance of RhoA to breast carcinogenesis and progression and compared the expression of Rho GTPases in breast tumors with that in normal tissue originating from the same individual. They found that RhoA protein expression is increased in tumors compared to the contiguous, non-involved tissue and that the relative level of RhoA protein significantly increased with histological grading, with the tumor proliferation index as determined by the quantification of MIB-positive cells, and the higher expression levels of RhoA mRNA and protein are associated with shorter disease-free and overall survival, indicating that RhoA may be a prognostic factor for this disease.

An important aspect of tumor treatment is the requirement for biomarkers that are easily measurable and can be subjected to repeated analysis for the purpose of monitoring the clinical stage of the disease, response to therapies, toxicities and other needs. Consequently, an ideal biomarker would preferably be available in easily accessible body fluids such as blood or urine, and in recent years the number of studies devoted to finding such biomarkers has increased significantly (49-50). Blood analysis of protein expression might accelerate the discovery of new diagnostic or prognostic biomarkers that in turn could be validated in a broader population. Various examples of such approaches are now available for the circulating tumor biomarkers utilized for the early diagnosis of several neoplasia, for the clinical efficacy of anticancer therapies and for monitoring patient follow-up (25). Serum proteomic profiles have also been suggested as a useful tool to monitor response to biological therapies (26). An interesting strategy has been developed that determines enzyme activity in human peripheral lymphocytes, and could be used to estimate the degree of sensitivity to specific cancer drugs (27). These biomarkers are even more useful if their production or release is associated with, or forms a part of specific tumor processes such as receptor presence or function, and proliferation or the potential to invade and form metastases. Hence, we undertook this study to characterize the expression in peripheral blood lymphocytes, which are easily accessible, of a protein that has been validated in biopsies as a tumor marker, RhoA (14-16,20-22,24,47), with the premise that positive results may further expedite the use of RhoA as a non-invasive diagnostic or prognostic biomarker for breast cancer.

In conclusion, the ability to measure RhoA protein expression in circulatory lymphocytes could greatly extend the uses of measuring RhoA protein in primary breast tumors, giving physicians advanced warning about the likely clinical outcome of the disease, and assisting them in decisions about whether to prescribe a more aggressive treatment regimen, such as chemotherapy, much earlier in the diagnostic process. Moreover, its measurement in circulatory lymphocytes could be substituted for the use of biopsies from a primary breast tumor and could be used to identify these more aggressive tumors at an earlier stage. Indeed, the sensitivity of this assay may permit both the early detection of aggressive tumors thus permitting more effective treatment and the follow-up of treatment effectiveness when tumor mass reduction could render biopsy difficult.

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