The Importance of Investigating Tumor Biology and Biomarkers in Pleural Effusions

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Abstract

Introduction: Pleural fluids can form in several diseases. In cancer patients particularly; diagnosing pleural fluids accurately can affect treatment and prognosis. Nevertheless, despite many studies carried out, it is difficult to distinguish malign-benign fluids and invasive processes are required. It was aimed in our study that biomarkers were analyzed by utilizing nucleic acid duplication method in pleural fluids attained by less invasive method as pleural puncture.

Methods: Of 54 patients who had applied to Chest Diseases Department and had been carried out pleural puncture for differential diagnosis, their pleura fluid samples were analyzed in Basic Oncology laboratory. Biomarkers were evaluated by examining expressions of 11 genes with PCR analysis.

Results: 45 (83%) out of 54 cases involved in the study had cancer diagnosis. 34% of malignancies were lung adenocarcinoma and 24% were breast cancer. In 53.4% of the cases with lung adenocarcinoma, TTF1 was observed; napsin A positivity was seen in 67% of cases with squamous cell carcinoma; both napsin A positivity and TTF1 positivity were determined in 67% of cases. ER was positive in 45% of cases with breast cancer. In the fluid analysis of 4 cases who were ER positive initially, received hormonotherapy and thereafter had pleural fluid workup result in cases (who receive treatment due to primary tumor or have been operated) assumed to have malignant pleurisy clinically with high likelihood is negative, it is recommended that advanced examination should be carried out (PCR analysis) and if the pleural fluid cytology workup result is positive, it will contribute to the diagnosis in analyzing biomarkers specific to tumor for the determination of primary tumor-pleural metastasis connection.

Discussion and Conclusion: When the results of our study were evaluated, we think that if pleural fluid cytology workup result in cases (who receive treatment due to primary tumor or have been operated) assumed to have malignant pleurisy clinically with high likelihood is negative, it is recommended that advanced examination should be carried out (PCR analysis) and if the pleural fluid cytology workup result is positive, it will contribute to the diagnosis in analyzing biomarkers specific to tumor for the determination of primary tumor-pleural metastasis connection.

Keywords: Pleural effusion; Biomarkers; Real-time PCR; Neoplasm; Metastasis; Tumor biology

Introduction

Pleural fluids are capable of forming in various diseases such as pneumonia, cancer, heart failure, tuberculosis [1]. In the differential diagnosis of malignant pleurises from these diseases, biopsy for primary tumor and if necessary surgical procedures (Chest Surgery with Video Aid (VATS), thoracoscopy) [2] and further cytologic examination (cell culture, immunohistochemistry, Polymerase Chain Reaction (PCR analysis)) are performed. Pleural fluid is known to contain plasma proteins together with inflammatory and proteins that epithelium and cancer cells release to the setting [3]. The fluid being rich in terms of proteins released and/or related to membrane and having specific biomarker for numerous diseases shows that it is an important material [4]. Since analyzing all new biomarkers includes all biological processes from gene expressions to protein changes, wide patient series and intensive molecular, proteomic studies are required. In our study, it was aimed that different biomarkers in pleural fluids with malign and benign cause should be examined with nucleic acid multiplication method (PCR analysis) and evaluated in terms of their contribution to diagnosis.

Materials and Methods

Our study was initiated with the decision of 2013/13-02 numbered ethical committee dated as 11.04.2013 and with 947-GOA protocol number and with 2015. KB. SAG. 007 numbered financial support by DEU Scientific Research Projects Coordination Unit. Informed consent was obtained from the cases concerning their wish to be included in the study. Pleural fluid samples were attained for differential diagnosis from 54 cases aged over 18 who had applied to Dokuz Eylül University Chest Diseases Department. Pleural fluid sample from 54 patients at Oncology Institute Basic Oncology Department laboratory was included in the study. During routine diagnostic procedures, some pleural fluid samples obtained by injector...
The molecular analysis of pleural fluids

Pleural fluid samples were portioned into 50 mL falcon tubes and serum part of the fluid was collected by centrifuging at 1600 rpm for 7 minutes. These serum samples were kept at -80°C until biomarker analysis. For total RNA isolation, cells were re-suspended in 200 μL Phosphate-buffered saline (PBS). 400 μL lysis and binding tampon were added and they were vortexed for 15 seconds and attached to collection tube with high pure filter. The sample was transferred to upper tube with filter and centrifuged for 15 seconds. The tube with filter was removed from the collection tube. The same tubes were attached again and wash tampon was added from primary-enzyme stock mixture prepared before. The sequences named as hormone response elements were added to the filter and incubated for 15 minutes in room temperature. 500 μL washing tampon I was added to the tube with filter and centrifuged at 8000 g for 15 seconds. After the fluid had been removed from the lower collection tube, the same tubes were attached again. 500 μL washing tampon were added to the tube with tampon II and centrifuged at 8000 g for 15 seconds. After the fluid accumulated in the lower collection tube had been removed, the same tubes were mounted again and 200 μL washing tampon II was added to the tube with filter and centrifuged at maximum speed. Later, the collection tube was disposed and the tube with filter was mounted to new 15 mL microcentrifuge tube. For RNA elution, 50-100 μL elution tampon was added to the filter tube. It was centrifuged at 8000 g for 1 minute. RNA that wanted to be acquired would be in the lower 1.5 mL microcentrifuge tube. RNA was kept at -80°C to be used in cDNA synthesis and then for Real-time PCR study and their amounts were measured at Qubit device. RNA amounts obtained from some samples were very low due to hypoplasia. Therefore, maximum amount of RNA that could be taken from RNA sample for cDNA conversion was obtained and cDNA synthesis was implemented with iScript™ cDNA Synthesis Kit (BioRad) as the producer firm explained. The quality of cDNA was tested with Real-time PCR reaction before it had been put into the array. The buffer stock including 0.1 μM 1X primary-enzyme mixture was prepared and kept at -20°C until its use. cDNA SYBR Green Master mix was prepared and it was put into 1 and 5 ml microcentrifuge tubes which were vortexed and distributed to every well of plates with 96 wells involving 9 μL. On top of the mixture, 1 μL was added from primary-enzyme stock mixture prepared before. The surface of PCR-array plate with 10 μL final volume in total was covered with seal, it was spun down at 2000 rpm for 1 minute and PCR was performed using High Pure PCR Clean/_extraction kit by Roche. PCR conditions: Primary concentration 0.1 μM, Programme: 95°C-10 s, 58°C-45 s (45 cycles). Attained Cq values were analyzed in “Qiagen Bioscience” programme. RNAs obtained were converted into cDNA and cDNAs were added to PCR mixture by avoiding DNA contamination carefully. Real-time PCR analysis became a kit prepared for effective inflammatory cytokines and receptors for immune response to be performed. In the data analysis, information was obtained regarding the expressions of indicators (Table 1). Biomarkers were arranged to determine the most common malignant pleural effusion reasons [5-7].

List of Biomarkers

<table>
<thead>
<tr>
<th>Thyroid Transcription Factor-1 (TTF1)</th>
<th>Calretinin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Napsin A</td>
<td>Synaptophysin (SYN)</td>
</tr>
<tr>
<td>Estrogen receptor (ER)</td>
<td>CD19</td>
</tr>
<tr>
<td>Chromogranin A (CgA)</td>
<td>CD3</td>
</tr>
<tr>
<td>Cytokeratin 7 (CK7)</td>
<td>Cytokeratin 5/6 (CK5/6)</td>
</tr>
<tr>
<td>Leukocyte common antigen (LCA-CD45)</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Biomarkers viewed with PCR array analysis.

Napsin A: It is an aspartic proteinase expressed from type II pneumocytes with proximal and distal renal tubule. It is expressed more than 80% in lung adenocarcinomas. It can be used as an addition to TTF-1 [11]. Napsin A is more sensitive than TTF-1 in primary lung cancer. If TTF-1 and Napsin A are positive together, lung adenocarcinoma is considered.

Calretinin: It is a protein dependent to 29 dDa calcium with EF-el structure [11,12]. It is expressed in peripheral and central nerve tissues and it has expressions in mesothelial cells. In some studies, calretinin is shown to be a positive marker for mesothelioma [13,14].

Estrogen Receptor (ER): Estrogen receptors are intracellular proteins and taken into cell depending on their concentration change and bind to hormone molecule selectively and form hormone-receptor complex. Activating hormone-receptor complex binds to short DNA sequences named as hormone response elements specifically in nucleus and performs transcription providing physiologic hormone activity [15]. In breast cancers, estrogen hormone has mutagenic and promotor effect. Mutagenic effect of estrogen hormone is organized by receptors and in culture its mutagenic effect in breast cancer cells carrying functional estrogen receptor was proved [16]. Particularly breast and endometrium carcinoma, prognostic importance of estrogen receptors was determined in a group of neoplastic diseases. ER (+) tumors responds to hormonal treatment and they show better prognosis. In healthy lung tissue and lung tumors, there is pretty much evidence that estrogen receptors are expressed and lung cancer responds to estrogens with proliferation. In samples obtained from patients with NSCLC, antibodies specific to Erβ demonstrate that these receptors are frequently expressed in NSCLC [17,18].

Chromogranin A (CgA): Chromogranins are acidic glycoproteins found in neurosecretory granules. They are 3 types; A, B and C. Chromogranin is commonly found in secretory vesicles in all endocrine, neuroendocrine and nervous system of CgA. CgA found from intercostal space were analyzed at basic oncology laboratory. No separate pleural fluid acquisition was applied to cases for this study.
together with peptide and amine hormones in these tissues where it takes charge in the regulation of secretory proteins is an acidic glycoprotein [19,20]. It is a general biomarker for CgA neuroendocrine cells depending on common expression in neuroendocrine system and high CgA levels are a helper in the determination of neuroendocrine tumors. It is established as positive in tumors such as gastric, pancreatic, small intestine and colorectal localization and small cell lung carcinoma, medullary thyroid carcinoma, neuroblastoma, pheochromocytoma, pituitary tumors, paraganglioma. In addition, it can be positive in such cancers as pancreatic adenocarcinoma, hepatocellular carcinoma, breast, colon, ovarian and prostate in small quantities [21].

**Synaptophysin (SYN):** It is a protein that forms a pore between synaptic vesicles composed of six polypeptides in synaptic vesicle membrane and plasma membrane. It is released from neuroendocrine tumors. CgA and SYN are frequently used as an indicator in the diagnosis of neuroendocrine tumors of lung. All typical and atypical carcinoids are CgA and/or SYN positive. However, small cell lung cancer must be kept in mind that it can show negativity at 25% rate. Gastroenteropathic neuroendocrine tumor (GEP-NET) cells express proteins as phenotypic SYN and CgA [22].

**CD19:** These cells are lymphocytes with 7-10 μm diameters. In addition to other indicators, they carry surface immunoglobulin, particularly IgM and IgD and at a low rate, IgG and IgA. They are determined as positive in CD19, B-cell lymphoma, leukemia and inflammations.

**CD3:** CD3 is expressed as heterodimer and accounts for 3 subunits called as α, β, γ and ζ subunit connected to them as homodimer. Every subunit includes immunoreceptor tyrosine-based activation motif (ITAM) with amino acid structure that places tyrosine residuals individually. ITAM tyrosines are a key for CD3 and ζ chains convey the signal. When α/β THR chains unite with peptide-MHC complex, they become phosphorylated. Following phosphorylation, ITAMs become a harbour for other proteins to commence signal cascade in T-cell activation. CD4 and CD8 proteins also play role in signal transmission [23]. It is determined as positive in CD3, lymphoma, leukemia and inflammations.

**Leukocyte common antigen (LCA-CD45):** It is positive in all lymphocytes; all lymphomas are stained positive. Follicular dendritic cells (FDC) are LCA positive [24].

**Cytokeratin 5/6 (CK5/6):** Cytokeratins are intermediate filaments and their real missions are to ensure cells to withstand mechanical stress. In humans, there are 20 different types of isotypes of cytokeratins. In squamous cell carcinomas, cytokeratin is 5/6 positive together with many keratin types. Cytokeratins with high molecular weight (HMW) like CK5/6 and CK14 are known as basal cytokeratins as they are determined in basal layer cells. These HMW cytokerines are established at 25% rate in grade 3 in situ breast lesions and invasive breast cancer at 2-18% rate. Therefore, this breast cancer group is named as a group showing basal/myoepithelial phenotype. In addition, basaloid group is used as a synonym of basal-like [25].

**Cytokeratin 7 (CK7):** It is coded by KRT 7 gene. Type II cytokeratin is composed of basic or neutral proteins during the differentiation of basic and stratified epithelium tissues. This type II cytokins cover the spaces of internal organs, gland channels and inner layer of blood vessels. In lung adenocarcinomas, CK 7 positivity is observed. It is evaluated with CK 20 [6,26].

The analysis of the data

Statistical analysis was carried out by using SPSS 22.0 programme. p<0.05 was statistically accepted as significant. In multigene analyses, Cp values were transformed into Ct values in the analysis sites of the firm concerned and compared with the gene expression profiles of test-based groups.

Results

In the study, certain amount of pleural fluid sample obtained for cytologic evaluation of 54 cases was reserved for the study. The demographical features of cases involved in the study were given in Table 2.

![Table 2: Demographical and clinical features of the cases.](image)

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>65.5 ± 11.58</td>
</tr>
<tr>
<td>Sex F/M (n)</td>
<td>27/27</td>
</tr>
<tr>
<td>Smoking +/- (n)</td>
<td>29/25</td>
</tr>
<tr>
<td>Smoking (packet/year)</td>
<td>53.9 ± 24.87</td>
</tr>
<tr>
<td>Cardiac disease</td>
<td>48.10%</td>
</tr>
<tr>
<td>Asthma</td>
<td>9.30%</td>
</tr>
<tr>
<td>COPD</td>
<td>44.40%</td>
</tr>
<tr>
<td>Pulmonary embolism</td>
<td>7.40%</td>
</tr>
</tbody>
</table>

Table 3: The distribution of cancer types of cases in malignant group.
The Importance of Investigating Tumor Biology and Biomarkers in Pleural Effusions

In our study, TTF1 was determined as positive in 53.4% of cases with lung adenocarcinoma. While in lung squamous cell carcinoma, TTF1 positivity was determined at 66.7% rate, TTF1 positivity was not determined in none of the extrapulmonary malignancies. Napsin A positivity was found as 45% in breast cancer cases, TTF1 positivity was not determined in none of breast cancer cases. Calretinin positivity was also determined in squamous cell carcinoma.

Discussion

The distribution of cases with malignant pleural fluid was as follows: 33.6% was lung adenocarcinoma, 24.4% was breast cancer and 6.7% was lymphoma and these rates are similar to those in literature. The most common malignant effusion causes in literature are 35% lung cancer, 23% breast cancer and 10% lymphoma [6].

In accordance with cytology results in our study, no malignant cells were seen in 44% of cases. Pleural fluids with no determined malignancy was thought to result from not having sufficient cells in samples that went to cytology and/or having pleural effusion regarding chemotherapy drugs, radiotherapy, infection.

TTF-1 was identified positive in 16 lung, 15 ovarian, 9 stomach, 8 colon and 8 breast cancers in 81% of cases. Nevertheless, TTF1 was not determined positive in extrapulmonary adenocarcinomas [26]. Kim et al. established TTF1 positive in 58% of 52 cases with lung adenocarcinoma and CDX2 positive in 30% colon and stomach cancers [27]. In our study, TTF1 was positive in 53, 4% of cases with lung adenocarcinoma and again it was determined positive in 66, 7% of cases with squamous cell carcinoma. However, having only 3 cases with squamous cell carcinoma is the limitation of the study. TTF1 was not determined positive in any of the malignancies apart from lung in our study.

<table>
<thead>
<tr>
<th>Cancer</th>
<th>PCR negative n (%)</th>
<th>PCR positive n (%)</th>
<th>Total n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung adenocarcinoma</td>
<td>5 (33.3)</td>
<td>10 (66.7)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>Small cell lung carcinoma</td>
<td>3 (50)</td>
<td>3 (50)</td>
<td>6 (100)</td>
</tr>
<tr>
<td>Lung carcinoid tumor</td>
<td>0</td>
<td>1 (100)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Lung squamous cell carcinoma</td>
<td>0</td>
<td>3 (100)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>2 (18.2)</td>
<td>9 (81.8)</td>
<td>11 (100)</td>
</tr>
<tr>
<td>Overian adenocarcinoma</td>
<td>2 (100)</td>
<td>0</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>0</td>
<td>3 (100)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>Stomach adenocarcinoma</td>
<td>1 (100)</td>
<td>0</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Extrapulmonary squamous cell carcinoma</td>
<td>0</td>
<td>1 (100)</td>
<td>1(100)</td>
</tr>
<tr>
<td>Renal cell carcinoma</td>
<td>1 (100)</td>
<td>0</td>
<td>1(100)</td>
</tr>
<tr>
<td>Colon adenocarcinoma</td>
<td>1 (100)</td>
<td>0</td>
<td>1(100)</td>
</tr>
</tbody>
</table>

**Table 4:** cDNA-SYBR green master mix mixture and array kit results.

**Table 5:** PCR array results were compared with routine cytologic evaluation results (p=0.003).
Liu et al. established both TTF1 and napsin A in 83% of cases with lung adenocarcinoma, only TTF1 as positive in 4%, only napsin A as positive in 9%. TTF1 and napsin A were not found positive in none of extrapolumary adenocarcinomas [28]. In our study, napsin A positivity was determined in 66, 7% in lung adenocarcinoma, in addition napsin A positivity was found as positive in 66% of cases with squamous cell carcinoma.

Ordóñez et al. established napsin A positivity in 29% in squamous cell carcinoma [29]. Having napsin A positivity at 45, 5% in breast cancer cases, on the other hand, having no TTF1 positivity in no cases with breast cancer contribute these two biomarkers to be used in the distinction of lung and extrapolumary adenocarcinomas.

In a study performed in a total of 77 cases including 22 reactive mesothelial cells, 26 malignant mesothelioma and 29 metastatic adenocarcinoma by Simsir et al. calretinin was determined positive at 27% in group involving reactive mesothelial cells, 58% in malignant mesothelioma group and 31% in metastatic adenocarcinoma group and no significant difference was found between groups [30]. In our study, having calretinin at 33% in breast cancer could bring to mind that reactive mesothelial cells were also present in the fluid. Powell et al. determined calretinin positive in 15% of cases with breast cancer [31]. Ordóñez et al. established calretinin positive in 40% in squamous cell lung carcinoma [32]. In our study, there is calretinin positivity in squamous cell carcinoma.

In a study performed among patients with lung and colorectal adenocarcinoma by Kummar et al. CK7+/CK20- was determined in 96% in primary lung cancer, 95% in metastatic lung cancer [33]. In our study, CK7 positivity was identified as 20% in lung adenocarcinoma, 33% in squamous cell lung carcinoma and 27% in breast cancer. Stopyra et al. found CK7+/CK20- phenotype significantly high in breast cancer and primary colon cancer [17].

In literature, the prognostic importance of estrogen receptor was identified notably in breast and endometrium carcinoma and in a group of neuroplastic illnesses. ER (+) tumours respond to hormonal therapy and show better prognosis. In healthy lung tissue and lung tumours, there is a great deal of evidence that estrogen receptors are expressed and they respond to lung cancer with estrogen proliferation. In samples obtained from patients with non-small cell lung cancer (NSCLC), antibodies specific to Erf display that these receptors are often expressed in NSCLC [18,34]. In our study, we determined ER as positive in 45% in many cases with breast cancer. In pleural effusion of four cases developed after chemotherapy, determining ER as negative and CK7 as positive gave rise to thought that ER-/CK7+ of an aggressive subgroup made a metastasis in pleura. ER positivity was established in squamous cell lung carcinoma in 33% in our study.

Tamiolakis et al. determined neuron-specific enolase (NSE) positivity in 100% in a series including 9 cases and TTF1, SYN and CgA positivity in 66% [35]. In carcinoid tumours in neuroendocrine tumour group, CgA positivity was reported in case series in literature. In our study, we established CgA positivity in a carcinoid tumour case. In 16, 7% of cases with small cell lung carcinoma, CgA positivity were present in our cases. Sobol et al. found a relationship between the increase in serum CgA levels and disease activation [36]. Leucocyte common antigen (LCA-CD-45) was shown to increase in human lymphomas and leukemia in various studies. In a study published by Nakano et al. the importance of CD45 was emphasized in the differentiation of hematopoietic system and cells [37].

In our study, LCA positivity was found in lymphomas in 33%. It was interpreted that 11% positivity rate could be related to the increase in inflammations in non-malignant group. Wick et al. determined LCA positivity in all 10 cases with colon and rectum small cell neuroendocrine carcinoma [38]. In the present study, LCA positivity was displayed in small cell lung carcinoma in 16%.

We determined CD3 as positive in 88% of cases in non-malignant group in our study. CD3 was thought to be associated with its being a lymphocyte derived biomarker. In all 3 lymphoma cases, we established CD3 as positive. In lung adenocarcinoma, breast cancer, small cell lung carcinoma; CD3 positivity was observed and it gives rise to thought that this metastatic process and inflammation accompany each other. Sebastian et al. reported that they received a response for intrapleural treatment in breast cancer cases when anti CD3 (Catumaxomab) was applied in the treatment of malignant pleural effusions [39].

In a study carried out by Dejmek et al. CK5/6 was determined positive in 7 out of 8 mesothelioma cases and 9 out of 11 benign pleural effusion cases. In addition, they found CK5/6 positive in 11 out of 17 cases with lung adenocarcinoma and 12 out of 32 extrapolumary adenocarcinomas; however, no significant difference was established between all groups. In pleural effusions related to NSCLC, it was reported that CK5 could be a prognostic marker [40]. Pu et al. found CK5/6 positive in all squamous cell cancers [41]. In our study, we determined CK5/6 positivity in 46% of cases with lung adenocarcinoma and in 66, 7% of lung squamous cell carcinomas. We established positivity in 63% of cases with breast cancer and in 1 case with extrapolumary squamous cell cancer.

CD19, CD21 and CD81 in B cells account for BHR (B Cell Receptor) complex similar to THR in T cells. CD18 was determined as positive in B cell-derived lymphoma, leukemia and inflammations. In our study, establishing CD19 positivity in 66% in non-malignant pleural effusions makes us think lymphocyte increase in effusion due to inflammation. We determined CD19 positive in all three lymphoma cases and in one extrapolumary squamous cell carcinoma.

In the diagnosis of neuroendocrine tumours of lung; CgA and SYN are most commonly used as a marker. All typical and atypical carcinoids are Cg A and/or SYN positive; nevertheless it is reported that they can display negativity in 25% in small cell lung cancer. In our study, SYN was determined positive in a lung carcinoid tumour case. SYN was positive in 33, 3% of our cases with small cell lung cancer.

**Conclusion**

As a result, PCR array and pleural effusions were determined to support malignancy in lung carcinoid tumour, lung squamous cell carcinoma, breast cancer, lymphoma and lung adenocarcinoma in our study. When cytology results and PCR array results were compared, it was found out that benign determination rate of pleural effusions was 66% in both methods and malignant determination level was 73%. However, that the distribution of tumour groups was not homogenous and the number of cases was less in some tumour types shows the limitation of the study.

When the results of ours study were evaluated; if cytologic workup result is negative in cases thought to be quite likely malignant pleurisy clinically (receiving treatment due to primary tumour or operated), we think that doing advanced research (primary cell culture, PCR analysis) could help diagnosis. In those whose cytologic workup result
is positive, though, displaying biomarkers specific to tumour in fluid is considered to provide additional contribution in determining primary tumour-pleural metastasis connection. In the light of this literature, multicentered studies involving more number of cases are required.

Patients’ Data Protection

Confidentiality of data

The authors declare that they have followed the protocols of their work center on the publication of patient data and that the patients included in the study have received sufficient information and given their informed consent in writing to participate in the study.

References

