

## **SHOX2 DNA Methylation Is a Tumour Marker in Pleural Effusions**

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**Abstract.** *Background:* The published sensitivity of cytological examination for malignant pleural effusions (MPE) ranges between 50% and 71%. The Epi proLung<sup>®</sup> BL Reflex Assay (Epigenomics AG, Berlin, Germany) has been reported as being highly sensitive and specific for lung cancer using bronchial aspirates. We hypothesize the assay to be of use in the detection of MPE. *Materials and Methods:* To test our hypothesis, we performed a retrospective cohort study on pleural effusion specimens of 1,270 patients (472 cases and 798 controls). The assay is based on quantification of methylated Short Stature Homeobox gene two (SHOX2) DNA in the specimen measured via multiplex real-time Polymerase Chain Reaction (PCR) on bisulfite-converted DNA. *Results:* Surprisingly, the assay detects metastases of lung cancer, as well as metastases of other malignant tumours. With a re-defined cut-off criterion, the test achieved a sensitivity of 39.5% with a specificity of 96.2%. *Conclusion:* This assay is able to detect MPE while not limited to the detection of lung cancer.

Although epidemiological studies are not available, the annual incidence of pleural effusion in the 27 European Union Member States (EU27) is estimated to be more than 1,600,000, based on the observed incidence in a well-defined region in central Bohemia and EU27 demographic data (1, 2). Each year, more than 1,360,000 patients are subjected to thoracentesis in the United States, even, if only the leading causes of pleural effusion are included (3). The reported share of patients with a malignant pleural effusion (MPE) is 25% to 45% (4, 5), highly depending on the observed population and study design, but nevertheless representing a

common problem. 15% of patients with a malignant tumour in a post-mortem series presented pleural effusion (6). The high probability of a malignant cause of pleural effusion necessitates thoracentesis and detailed cytological analysis in every case. Non-malignant aetiologies of pleural effusion include congestive heart failure, infection, pulmonary embolus or infarction, cirrhosis and collagen disease (4). Malignant tumours presenting with MPE include cancer of the lung (approximately one third of the MPE), breast, ovary, gastrointestinal tract, mesothelioma and lymphoma (4, 7). In the vast majority of cases, MPE is a sign of metastasized cancer, meaning that a curative therapy is no longer reasonable. This necessitates the accurate diagnosis of MPE and avoidance of invasive diagnostic procedures in order to prevent further stress for the patient.

Pleural fluid cytology for the diagnosis of MPE is the standard procedure, with a high specificity (97% to 100%) but limited sensitivity (50% to 71%) (5). In addition, some pleural fluid specimens cannot be diagnosed cytologically as being definite tumour-positive or -negative (8), leading to an equivocal result in 5.8% of cases in a previous study from our Department (5). Thus, there is a need for additional methods, preferentially on the same pleural fluid, again to prevent repeated diagnostic efforts and harm of the patient. There are numerous different strategies to enhance the diagnostic yield of cytology (9, 10). These strategies include morphological, DNA, RNA, methylation and protein analyses (including immunocytochemistry), electron microscopy, analysis of the argyrophilic nucleolar organizer regions (AgNOR), flow cytometry, image cytometry, fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR)-based assays.

Aberrant DNA methylation plays an important role in carcinogenesis (11) and is purported to be a valuable cancer biomarker (12, 13). Epigenetic cancer biomarkers are independent of classical morphology and thus exhibit great potential to overcome the limitations of cytology. The usefulness of epigenetic cancer biomarkers has been shown for body fluids, such as bronchial aspirate (14), sputum (15, 16), plasma (17) and serum (18). Short Stature Homeobox gene two (SHOX2) DNA methylation in particular has been

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shown to occur at a high prevalence (96%) in carcinomas of the lung, especially in squamous cell carcinomas and small cell carcinomas, compared to matched morphologically normal adjacent tissues from the same patients (19). In addition, *SHOX2* DNA methylation has been proven to be a useful biomarker in bronchial aspirates to detect lung cancer, even subsequent to tumour-negative or equivocal cytology. The ability of *SHOX2* methylation was successfully tested and validated in two case-control studies, including 523 and 250 patient samples, respectively, with high sensitivity (78%) and specificity (96%) (20, 21). This led to the development of the Epi proLung® BL Reflex Assay (Epigenomics AG, Berlin, Germany), an *in vitro* diagnostic test kit to aid pathologists in the diagnosis of lung cancer. *SHOX2* encodes for a homeo-domain-transcription factor and has been identified as highly homologous to the short stature homeobox gene *SHOX*. Both genes are involved in skeletogenesis and heart development (22-24).

The objective of our current study was to analyze the diagnostic value of testing *SHOX2* DNA methylation in a retrospective cohort of pleural effusions sent to the Department of cytopathology. Furthermore, we wanted to confirm if aberrant *SHOX2* DNA methylation is specific to lung carcinoma, as suspected in recent studies (19-21, 25), or if the *SHOX2* DNA methylation marker may also be of use to identify other tumour entities.

## Materials and Methods

**Study design.** The study was a retrospective cohort study, and the pleural effusions were collected before the examination started. The specimens were examined blinded for the reference standard, and the reference standard was obtained blinded for the examination data. The study was approved by the Local Ethics Committee (#3383).

**Study inclusion criteria.** First-time pleural effusion samples of 1,617 patients were submitted to the Department of cytopathology between January 2010 and August 2011. Only specimens from hospitals submitting more than 20 specimens during the mentioned period were included.

**Cytological diagnosis.** The submitted native pleural effusion fluid (usually about 50 ml) was processed according to standard laboratory protocols. In brief, the specimens were centrifuged at 670 ×g for 5 min. The supernatant was transferred to a new container. Eight smears were prepared with aliquots of the resuspended pellet. Three of them were stained according with May-Grünwald-Giemsa. The other five smears were fixated by the Delaunay method and stained according to Papanicolaou. A final cytological diagnosis was made by experienced cytopathologists, including diagnostic immunocytochemistry, DNA image cytometry or AgNOR analysis, when necessary.

**Sample preparation.** Remaining material from the pellet was preserved in Saccomanos fixative [50% (v/v) ethanol, 2% (w/v) polyethylene glycol 1,500, and 60 mg/l rifampicin] and stored at

4°C until further use. DNA extraction and bisulfite conversion were performed with 80 µl of the fixed sample using the Epi proLung® BL DNA Preparation Kit (Epigenomics AG). The samples were prepared in groups of 22 samples, together with positive and negative controls from the Epi proLung® BL Work Flow Control Kit (Epigenomics AG).

**Real-time PCR.** The real-time PCR was performed using the Epi proLung® BL real-time PCR Kit (Epigenomics AG), and a Applied Biosystems® 7500 Fast real-time PCR instrument (Life Technologies Corporation, Carlsbad, USA). The Epi proLung® BL real-time PCR Kit was designed to detect lung cancer in bronchial lavage specimens. The PCR is a multiplex real-time PCR with two targets. The first target is a methylation-sensitive sequence of *SHOX2*. The second target is a methylation independent sequence of the beta-actin gene (*ACTB*). According to the PCRkit manual, the PCR was performed in triplets of each sample, positive control, negative control and calibrator provided by the work flow kit. A PCR run was valid if the following criteria were met: two out of three replicates of the negative control with a cycle threshold (Ct) value  $Ct(SHOX2) \geq 38$  and a  $Ct(ACTB)$  of between 28 and 37; all replicates of the positive control with a  $Ct(SHOX2) \leq 37$  and a  $Ct(ACTB) \leq 31$ ; two or more of the calibrator replicates with  $Ct(ACTB) \leq 32$  and a  $\Delta Ct_{Cal} [=Ct(SHOX2)-Ct(ACTB)]$  in the range of -2.6 and -0.6. The measurement of a sample was valid if the PCR run was valid and if more than one of the three replicates had a  $Ct(ACTB)$  of 29 or less.

**Data and statistical analysis.** For each valid sample, a relative methylation value was determined using the  $\Delta\Delta Ct$  method (26) in which the frequency of methylation is inversely proportional to the  $\Delta\Delta Ct$  value and can be approximated using the formula:  $Methylation (\%) = 100\% / 2^{\Delta\Delta Ct}$ . According to the Epi proLung® BL kit manual, a  $\Delta\Delta Ct$  equal or below 9.5 would define the sample as being *SHOX2* methylation-positive and therefore as lung cancer-positive. The cut-off had been determined for bronchial lavage samples (20). Examining pleural effusions, we reviewed this cut-off and used the receiver operating characteristic (ROC) to determine an optimal cut-off for pleural effusions. Invalid measured samples and samples with indistinct reference standard were excluded from this analysis. All statistical data were calculated using R (version 3.0.1) (27). If not otherwise stated, significance was calculated using the “Pearson’s Chi-square Test for count data” without the Yates’ continuity correction. The level of significance was set at  $p=0.05$ . In cases of multiple testing, the individual level of significance for each test was adjusted using the Bonferroni correction. The calculation of the ROC, the 95% confidence interval (CI) for the ROC and the area under the ROC curve (AUC) was carried out using the R package “pROC” (version 1.5.4) (28). Contingency tables were used to analyse the categorical data provided by both index tests and reference standard to determine the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of cytology and *SHOX2* methylation. 95% Confidence intervals (CI) are given, using the R package “bdpv” (version 1.0) (29).

**Reference standard.** Clinical follow-up was obtained by review of the patients’ medical files. A reference standard (malignant pleural tumour present/absent) was compiled for each patient. Special emphasis was placed on the final clinical decision regarding the

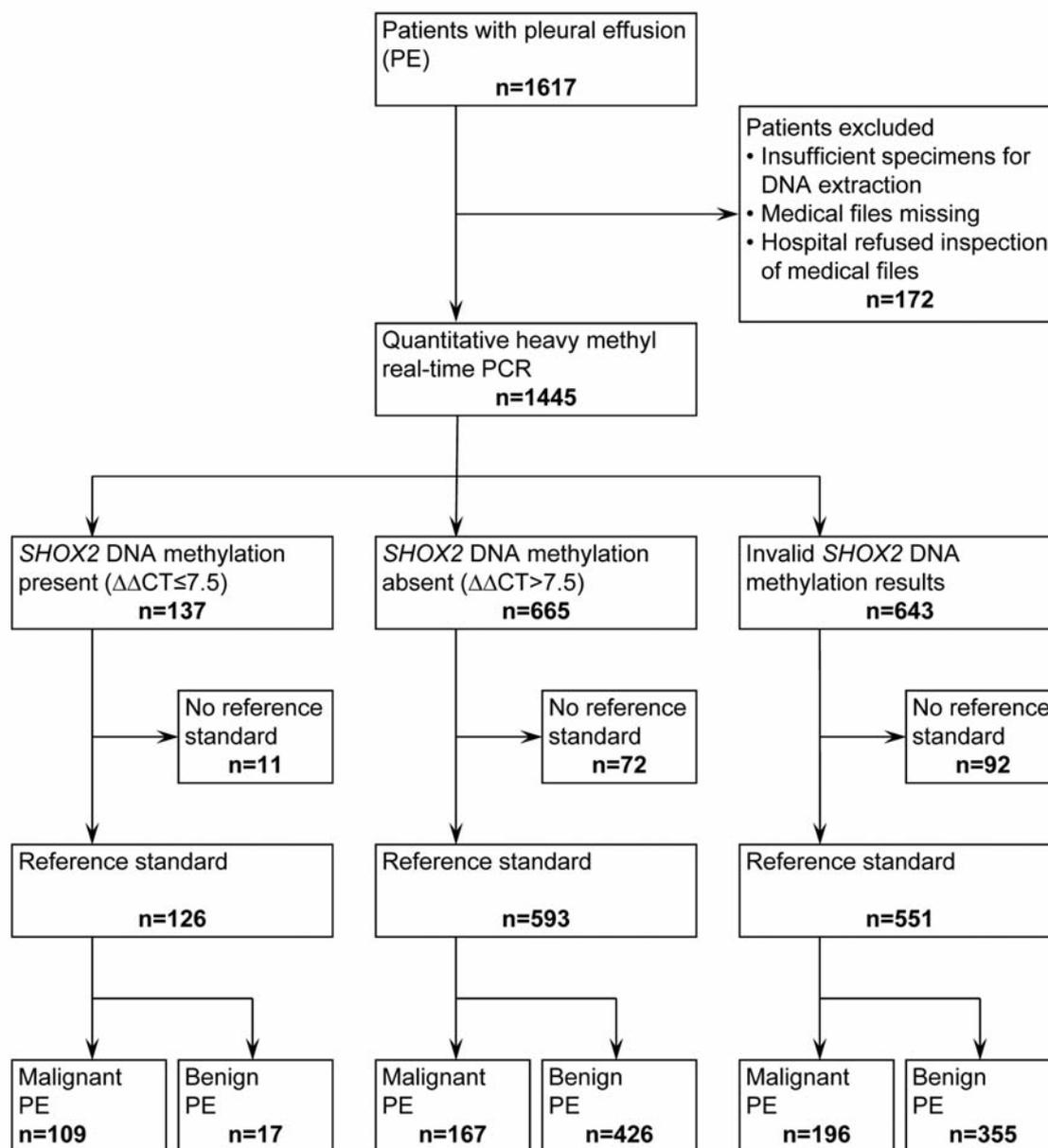


Figure 1. The figure presents the data of our study on pleural effusion (PE) in a flow chart according to the STARD initiative (30).

underlying cause of the pleural effusion, existence of a malignant tumour from the patients' history (confirmed by histology or adequate tumour therapy) and tumour exclusion by medical imaging.

## Results

The flow of the patients through the study is presented in a Standards for Reporting of Diagnostic Accuracy (STARD) diagram (Figure 1) (30). Pleural effusion samples of 1,617 patients were collected. Out of these, 172 specimens were excluded from the study either because the material was not sufficient for DNA extraction (no visible pellet after

centrifugation), or because the follow-up data were inaccessible due to missing patient's medical files or rejection of the hospital's participation in the study. In the case of 175 patients with tumours, the reference standard could not be definitively determined because of comorbidity. Therefore, the follow-up reference standard was evaluated regarding 1,270 out of the total of 1,445 patients. The clinical characteristics of the 1,270 patients are presented in Table I.

All 1,445 pleural effusions were analysed using the Epi proLung® BL Reflex Assay. A valid measurement (according to the assay's manual) was achieved for 802 specimens

Table I. Clinical characteristics of the patient population. The table presents the data of 1,270 patients with evaluable reference standard.

| Frequency                              | Benign pleural effusion <sup>a</sup> (n=798) |             | Malignant pleural effusion <sup>a</sup> (n=472) |             |
|--|--|-------------|---|-------------|
|  | Female<br>333                                | Male<br>465 | Female<br>240                                   | Male<br>232 |
| Age, years                             |  |             |   |             |
| Average                                | 73.4   | 70.0        | 67.3  | 67.7        |
| Standard deviation                     | 15.6   | 14.4        | 13.1  | 11.7        |
| Median                                 | 77   | 73          | 69  | 70          |
| Range                                  | 4-97   | 2-96        | 20-96   | 3-90        |
| Benign condition <sup>b</sup>          |  |             |   |             |
| Infectious disease <sup>c</sup>        | 171  | 255         | 19  | 33          |
| Congestive heart failure               | 260  | 301         | 26  | 17          |
| Gastrointestinal disease <sup>d</sup>  | 14   | 35          | 4   | 5           |
| Trauma/haemothorax                     | 18   | 21          | 1   |             |
| Renal failure                          | 99   | 147         | 21  | 26          |
| Post-surgery                           | 31   | 31          | 5   | 7           |
| Collagen vascular disease <sup>e</sup> | 8  | 8           | 2   | 2           |
| Other <sup>f</sup>                     | 113  | 143         | 39  | 61          |
| Malignant condition <sup>g</sup>       |  |             |   |             |
| Lung cancer                            | 1  | 4           | 71  | 125         |
| Breast cancer                          | 14   | 1           | 101   |             |
| Gastrointestinal cancer <sup>h</sup>   | 6  | 10          | 30  | 49          |
| Lymphoma/leukaemia <sup>i</sup>        | 10   | 4           | 21  | 21          |
| Mesothelioma                           |  |             | 2   | 9           |
| Other <sup>j</sup>                     | 13   | 31          | 44  | 67          |
| Cancer therapy <sup>k</sup>            |  |             |   |             |
| Surgery                                | 29   | 34          | 114   | 81          |
| Radiotherapy                           | 11   | 7           | 80  | 66          |
| Chemotherapy                           | 17   | 16          | 160   | 150         |
| Pleurodesis                            |  |             | 48  | 44          |
| Stem cell therapy                      |  | 1           | 1   | 8           |

Unless otherwise stated, the data are presented as frequencies. <sup>a</sup>According to reference standard criteria. <sup>b</sup>Comorbidities are frequent. <sup>c</sup>Pneumonia (n=331); empyema (n=38); mediastinitis (n=1); pleuritis (n=35); sepsis (n=60); tuberculosis (n=13). <sup>d</sup>Liver cirrhosis (n=46); pancreatitis (n=12). <sup>e</sup>Lupus erythematosus (n=7); rheumatoid arthritis (n=13). <sup>f</sup>Amyloidosis (n=1); sarcoidosis (n=6); chronic obstructive pulmonary disease (n=199); emphysema (n=24); myocardial infarction (n=22); cardiac arrhythmia (n=104). <sup>g</sup>Some patients developed more than one tumour. <sup>h</sup>Cholangiocarcinoma (n=4); colorectal carcinoma (n=38); gastric carcinoma (n=20); pancreatic carcinoma (n=21); oesophageal carcinoma (n=12). <sup>i</sup>Acute myeloid leukaemia (n=4); chronic myelogenous leukaemia (n=7); Hodgkin's lymphoma (n=3); myelodysplastic syndrome (n=6); precursor and mature B- and T-cell neoplasms (n=35); unclassified lymphoma (n=1). <sup>j</sup>Carcinoma (n=6) and melanoma (n=2) of the skin; carcinoma of the oropharynx (n=6), larynx (n=6), salivary gland (n=2), thyroid (n=4), vulva (n=2), uterine cervix (n=2), endometrium (n=1), ovary (n=23), prostate (n=28), urinary system (n=23), kidney (n=17); hepatocellular carcinoma (n=7); primary peritoneal carcinoma (n=1); Kaposi sarcoma (n=2); rhabdomyosarcoma (n=1); synovial sarcoma (n=1); extraskeletal Ewing's sarcoma (n=1); uterine sarcoma NOS (n=1); medulloblastoma (n=1); paraganglioma (n=1); cancer of unknown primary (n=17). <sup>k</sup>Repeated therapies are only mentioned once.

(55.5%), leaving 643 specimens (44.5%) with invalid measurements. The Ct(*ACTB*) criterion was the most frequent reason for invalid measurements. The reference standard was evaluable for 719 out of the 802 specimens with valid measurements, and for 551 out of the 643 of those with invalid measurements. Of the 1,270 specimens with an evaluable reference standard, the 719 patients with valid test results were compared to the 551 with invalid test results for the parameters gender, follow-up result, cytological examination result and age. Both groups were not significantly different in their distribution between the sexes ( $p=0.80$ ), their follow-up result ( $p=0.30$ ), cytological examination result ( $p=0.63$ ), or in the distribution of their age ( $p=0.02$ ) (acceptable level of significance,  $p<0.0125$  according to Bonferroni correction).

The next step was comparing the 719 valid *SHOX2* methylation measurements with the corresponding follow-up diagnoses. The reference standard defined 276 of these as positive and 443 as negative. With the cut-off criterion of  $\Delta\Delta Ct \leq 9.5$  as stated by the assay, 138 of the 276 positive samples had a correct positive test result (sensitivity=50.0%, 95% CI=43.9%-56.1%). Out of the 443 negative samples, there were 373 with a correct negative test result (specificity=84.2%, 95% CI=80.5%-87.5%). The cut-off criterion of the assay had been determined for bronchial aspirates (20, 21). In order to adjust the cut-off for use with pleural effusions, the cut-off criterion was optimized by analysing the test with its receiver operating characteristic (ROC) curve. The area under the ROC curve (AUC) was 0.72 (95% CI=0.68-0.76) and the new cut-off determined as an optimization between sensitivity and specificity was therefore  $\Delta\Delta Ct \leq 7.5$ . With this cut-off, the test achieved a sensitivity of 39.5% (109/276) (95% CI=33.7%-45.5%) and a specificity of 96.2% (426/443) (95% CI=93.9%-97.7%).

The cytological examination of these specimens achieved 52.5% (145/276) (95% CI=46.5%-58.6%) sensitivity and 99.8% (442/443) (95% CI=98.7%-100.0%) specificity. A combined evaluation of the cytological examination and the *SHOX2* assay was carried out as follows: All cytological tumour-positive diagnoses were counted as positive, and no additional *SHOX2* assay was performed. All tumour-negative and tumour-suspicious cytological diagnoses were followed by an additional *SHOX2* assay. This led to 58.0% (160/276) (95% CI=51.9%-63.9%) sensitivity and 96.2% (426/443) (95% CI=93.9%-97.7%) specificity. These data are presented in Table II together with the positive and negative predictive value (PPV, NPV) of these tests. With a cut-off of  $\Delta\Delta Ct \leq 3.5$ , the sensitivity was 17.8% (49/276) (95% CI=13.4%-22.8%) without any false-positive results (specificity=100% (443/443) (95% CI=99.2%-100.0%).

In addition to lung carcinomas which were identified as positive, the *SHOX2* assay also detected cancer of other origins, no matter which of the presented cut-off values were used. Table III presents the sensitivity to different malignant

Table II. Sensitivity and specificity of the short stature homeobox gene-two (*SHOX2*) methylation assay and the cytological examination. The table shows the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for the examination of 719 valid measured specimens using *SHOX2* methylation assay, cytological examination and a combination of cytological examination and the *SHOX2* assay. The data are presented as the percentage (frequency) (95% CI).

|             | <i>SHOX2</i> assay             |                                | Cytological examination        | Cytology+ <i>SHOX2</i> assay ( $\Delta\Delta Ct \leq 7.5$ ) |
|-------------|--------------------------------|--------------------------------|--------------------------------|---|
|             | ( $\Delta\Delta Ct \leq 9.5$ ) | ( $\Delta\Delta Ct \leq 7.5$ ) |                                |   |
| Sensitivity | 50.0% (138/276) (43.9%-56.1%)  | 39.5% (109/276) (33.7%-45.5%)  | 52.5% (145/276) (46.6%-58.4%)  | 58.0% (160/276) (51.9%-63.9%)                               |
| Specificity | 84.2% (373/443) (80.5%-87.5%)  | 96.2% (426/443) (93.9%-97.7%)  | 99.8% (442/443) (98.7%-100.0%) | 96.2% (426/443) (93.9%-97.7%)                               |
| PPV         | 66.3% (138/208) (60.7%-71.6%)  | 86.5% (109/126) (79.7%-91.3%)  | 99.3% (145/146) (95.3%-99.9%)  | 90.4% (160/177) (85.4%-93.8%)                               |
| NPV         | 73.0% (373/511) (70.5%-75.4%)  | 71.8% (426/593) (69.8%-73.8%)  | 77.1% (442/573) (74.9-79.3%)   | 78.6% (426/542) (76.2%-80.9%)                               |

tumour entities as related to the cut-off of  $\Delta\Delta Ct \leq 7.5$ . Some patients (n=35) had more than one malignant tumour, possibly metastasized to the pleural cavity, hence they were excluded from subsequent evaluation.

The frequency of detected lung cancer was not significantly different from the frequency of detected malignant tumours of other origin ( $p=0.85$ ).

Of the 17 patients with a false-positive *SHOX2* result, three had a malignant tumour (Hodgkin lymphoma, basalioma and salivary gland carcinoma) in their history besides benign pleural effusion according to the follow-up. All three of them died within 15 days after we received their specimens. Two more of the 17 patients died within 18 days after we received their specimens. The remaining 12 patients did not exhibit development of a malignant tumour during the follow-up period which differs between 2 and 18 months. The 17 patients had the following spectrum of relevant benign diseases (sorted by frequency): congestive heart failure (n=7), cardiac congestion (n=7), advanced renal disease (n=3), pneumonia (n=3), sepsis (n=3) and post myocardial infarction (n=2). These diseases were also present in the group of the patients who were *SHOX2*-negative.

## Discussion

The performance of the *SHOX2* methylation assay when used for its intended use on bronchial aspirates was 81% (95% CI=71%-89%) sensitivity and 95% (95% CI=88%-99%) specificity (20). As expected, the test performance on pleural effusions with the same cut-off criterion was different: its sensitivity was 50.0% (95% CI=43.9%-56.1%) and its specificity was 84.2% (95% CI=80.5%-87.5%). For both studies, only the valid measured specimens were evaluated. The most probable reason for these highly different results is the difference in the tested materials themselves: pleural effusion fluid is a very different source of analyte from bronchial lavage, especially in regard to its composition. Pleural effusion is a serous fluid, seldom mucoid, in most cases sterile, frequently with a haemorrhagic component and usually contains

Table III. Performance of the short stature homeobox gene-two (*SHOX2*) methylation assay for different types of malignant tumours. The frequency and percentage of *SHOX2*-positive and -negative results for different tumour types, with a cutoff of  $\Delta\Delta Ct \leq 7.5$ , and the frequency of these tumour types are shown. Only patients with positive reference standards and only one known cancer origin were evaluated.

|                         | <i>SHOX2</i> |           | Frequency |
|-------------------------|--------------|-----------|-----------|
|                         | Positive     | Negative  |           |
| Lung cancer             | 44 (40%)     | 67 (60%)  | 111       |
| Breast cancer           | 16 (55%)     | 13 (45%)  | 29        |
| Mesothelioma            | 4 (67%)      | 2 (33%)   | 6         |
| Lymphoma/leukaemia      | 6 (26%)      | 17 (74%)  | 23        |
| Gastrointestinal cancer | 11 (37%)     | 19 (63%)  | 30        |
| Other                   | 13 (31%)     | 29 (69%)  | 42        |
| Total                   | 94 (39%)     | 147 (61%) | 241       |

mesothelial cells. In contrast, bronchial aspirates are aspirated mucoid fluids, often mixed with rinsing fluids (lavage), frequently with bacterial and macrophagial background, rarely with a haemorrhagic component and usually contain epithelial cells. In contrast to the recommendations of the user's-manual for the Epi proLung<sup>®</sup> test, the Saccomannos-fixed material used in this study was stored at 4°C for a maximum of two years. However, this should not be of great influence as the Saccomannos-fixed material used for the previous studies with this test on bronchial lavage was stored at room temperature for 12 years (20). The AUC regarding this study was 0.72 and as such between the value of 0.94 achieved by the *SHOX2* methylation assay on bronchial aspirates (20) and the that of 0.63 evaluated with methylation-sensitive high resolution melting analysis of 97 non-small cell lung cancer samples and corresponding normal lung tissue (31).

The cut-off criterion of the *SHOX2* methylation assay established for the diagnosis of bronchial lavage is not directly transferable to the diagnosis of pleural effusions as

shown above. Therefore, two new cut-off criteria were considered. These criteria were the  $\Delta\Delta Ct \leq 7.5$  criterion, which provides an optimum balance of sensitivity and specificity, and the  $\Delta\Delta Ct \leq 3.5$  criterion, with a very high specificity at the expense of sensitivity.

The *SHOX2* methylation assay was able to achieve a valid measurement in 719 cases out of 1,270 specimens with evaluable reference standard. Consequently, 43.4% (551/1,270) measurements were declared invalid. This matches the validation study by Dietrich and co-workers on bronchial aspirates, who had 38.4% (96/250) invalid results, most probably due to DNA degradation of the archival specimens, applying the validity criteria provided by the assay (20). The valid and invalid results in the presented study are not significantly different in the distribution of the follow-up result, cytological diagnosis, sex and age of the patients. Accordingly, we conclude that the above-mentioned crucial parameters do not influence the consideration of measurement of a specimen as being valid or invalid.

Schmidt and co-workers measured *SHOX2* DNA methylation on 523 bronchial aspirates from 281 lung cancer patients and 242 controls, and stated that increased *SHOX2* methylation is highly specific for the detection of lung cancer, and that this specificity is increased by the origin of their material (21). Kneip and co-workers investigated *SHOX2* DNA methylation as a biomarker for the detection of malignant lung disease in plasma of patients with lung cancer (25). Using the same assay, they achieved a sensitivity of 60% (95% CI=53-67%) and a specificity of 90% (95% CI=84-94%). Their collective consisted of 188 lung tumour cases and 155 controls. Of the controls, 151 were samples from healthy individuals or patients with a benign lung disease, and the remaining four samples were from patients who had carcinoma of the prostate. In contrast to our study on pleural effusions, patients with other types of malignant tumours were not included. Our results on pleural effusions demonstrate that the *SHOX2* methylation marker is able to detect lung cancer and other tumour types. Furthermore, the detection rates regarding lung cancer and tumours of other origin do not significantly differ. Therefore, we conclude that the ability of the *SHOX2* methylation marker when used on pleural effusions is not limited to the detection of lung cancer.

Comparing the results of the cytological examination to the results of the *SHOX2* methylation assay, the cytological examination provides higher sensitivity and higher specificity. Therefore, the *SHOX2* methylation assay is not suitable for replacing the cytological examination. The evaluated combination of both methods is also not advisable since it has an increased sensitivity at the cost of reduced specificity. Using the stricter cut-off ( $\Delta\Delta Ct \leq 3.5$ ) for the combination of both methods would not lead to any increase in sensitivity nor in specificity. Nevertheless, the assay could be of use. The cytological examination requires a network of

very well-trained pathologists. This infrastructure may not be established in every region. The *SHOX2* methylation assay is a PCR-based method, and can as such be up-scaled to a high-throughput method. As a high-throughput method, its use could be centralized until a network of pathologists is established.

Further applicability of the *SHOX2* methylation assay for pleural effusions, using the  $\Delta\Delta Ct \leq 3.5$  criterion to provide a sensitivity of 17.8% with a specificity of 100%, could be the combination with other methylation-based tumour markers to enhance the sensitivity of detecting MPE. Admittedly 17.8% sensitivity is very low, but in order to maintain high specificity, it is crucial that all of the combined methods provide high specificity. These other markers could, for example, be some of those examined by Brock *et al.* (32). They achieved 67% sensitivity (100% specificity) on a prospective cohort of 31 patients with pleural effusions applying methylation-specific PCR with a marker panel of eight different genes [retinoic acid receptor-beta (*RARB*), adenomatous polyposis coli (*APC*), breast cancer 1 early onset (*BRCA1*), methylguanine-DNA methyltransferase (*MGMT*), cellular retinol-binding protein 1 (*CRBP1*), fragile histidine triad (*FHIT*), ras association domain family member 1 (*RASSF1A*) and cyclin-dependent kinase inhibitor 2A (*p16*)].

## Conclusion

The *SHOX2* DNA methylation assay is a promising method for the detection of a malignant cause of pleural effusion. The lung cancer specificity reported for bronchial aspirates, however, is not confirmed for pleural effusions.

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## References

- 1 European Commission: European social statistics - edition 2013. Publications Office of the European Union, Luxembourg, 2013.
- 2 Marel M, Zrustova M, Stasny B and Light RW: The incidence of pleural effusion in a well-defined region. Epidemiologic study in central Bohemia. *Chest* 104: 1486-1489, 1993.
- 3 Light RW: Clinical practice. Pleural effusion. *N Engl J Med* 346: 1971-1977, 2002.
- 4 Bedrossian CWM: Malignant Effusions: A Multimodal Approach to Cytologic Diagnosis: Igaku-Shoin, New York, 1994.
- 5 Motherby H, Nadjari B, Friegel P, Kohaus J, Ramp U and Bocking A: Diagnostic accuracy of effusion cytology. *Diagn Cytopathol* 20: 350-357, 1999.

- 6 Rodriguez-Panadero F, Borderas Naranjo F and Lopez Mejias J: Pleural metastatic tumours and effusions. Frequency and pathogenic mechanisms in a post-mortem series. *Eur Respir J* 2: 366-369, 1989.
- 7 Antony VB, Loddenkemper R, Astoul P, Boutin C, Goldstraw P, Hott J, Rodriguez Panadero F and Sahn SA: Management of malignant pleural effusions. *Eur Respir J* 18: 402-419, 2001.
- 8 Bedrossian CW: Diagnostic problems in serous effusions. *Diagn Cytopathol* 19: 131-137, 1998.
- 9 Mohanty SK and Dey P: Serous effusions: diagnosis of malignancy beyond cytomorphology. An analytic review. *Postgrad Med J* 79: 569-574, 2003.
- 10 Sriram KB, Relan V, Clarke BE, Duhig EE, Yang IA, Bowman RV, Lee YC and Fong KM: Diagnostic molecular biomarkers for malignant pleural effusions. *Future Oncol* 7: 737-752, 2011.
- 11 Ting AH, McGarvey KM and Baylin SB: The cancer epigenome-components and functional correlates. *Genes Dev* 20: 3215-3231, 2006.
- 12 Esteller M: Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet* 8: 286-298, 2007.
- 13 Laird PW: The power and the promise of DNA methylation markers. *Nat Rev Cancer* 3: 253-266, 2003.
- 14 Schmiemann V, Bocking A, Kazimirek M, Onofre AS, Gabbert HE, Kappes R, Gerharz CD and Grote HJ: Methylation assay for the diagnosis of lung cancer on bronchial aspirates: a cohort study. *Clin Cancer Res* 11: 7728-7734, 2005.
- 15 Belinsky SA, Liechty KC, Gentry FD, Wolf HJ, Rogers J, Vu K, Haney J, Kennedy TC, Hirsch FR, Miller Y, Franklin WA, Herman JG, Baylin SB, Bunn PA and Byers T: Promoter hypermethylation of multiple genes in sputum precedes lung cancer incidence in a high-risk cohort. *Cancer Res* 66: 3338-3344, 2006.
- 16 Palmisano WA, Divine KK, Saccomanno G, Gilliland FD, Baylin SB, Herman JG and Belinsky SA: Predicting lung cancer by detecting aberrant promoter methylation in sputum. *Cancer Res* 60: 5954-5958, 2000.
- 17 Hsu HS, Chen TP, Hung CH, Wen CK, Lin RK, Lee HC and Wang YC: Characterization of a multiple epigenetic marker panel for lung cancer detection and risk assessment in plasma. *Cancer* 110: 2019-2026, 2007.
- 18 Benlloch S, Galbis-Caravajal JM, Martin C, Sanchez-Paya J, Rodriguez-Paniagua JM, Romero S, Mafe JJ and Massuti B: Potential diagnostic value of methylation profile in pleural fluid and serum from cancer patients with pleural effusion. *Cancer* 107: 1859-1865, 2006.
- 19 Schneider KU, Dietrich D, Fleischhacker M, Leschber G, Merk J, Schaper F, Stapert HR, Vossenaar ER, Weickmann S, Liebenberg V, Kneip C, Seegebarth A, Erdogan F, Rappold G and Schmidt B: Correlation of *SHOX2* gene amplification and DNA methylation in lung cancer tumors. *BMC Cancer* 11: 102, 2011.
- 20 Dietrich D, Kneip C, Raji O, Liloglou T, Seegebarth A, Schlegel T, Flemming N, Rausch S, Distler J, Fleischhacker M, Schmidt B, Giles T, Walshaw M, Warburton C, Liebenberg V and Field JK: Performance evaluation of the DNA methylation biomarker *SHOX2* for the aid in diagnosis of lung cancer based on the analysis of bronchial aspirates. *Int J Oncol* 40: 825-832, 2012.
- 21 Schmidt B, Liebenberg V, Dietrich D, Schlegel T, Kneip C, Seegebarth A, Flemming N, Seemann S, Distler J, Lewin J, Tetzner R, Weickmann S, Wille U, Liloglou T, Raji O, Walshaw M, Fleischhacker M, Witt C and Field JK: *SHOX2* DNA methylation is a biomarker for the diagnosis of lung cancer based on bronchial aspirates. *BMC Cancer* 10: 600, 2010.
- 22 Blaschke RJ, Hahurij ND, Kuijper S, Just S, Wisse LJ, Deissler K, Maxelon T, Anastassiadis K, Spitzer J, Hardt SE, Scholer H, Feitsma H, Rottbauer W, Blum M, Meijlink F, Rappold G and Gittenberger-de Groot AC: Targeted mutation reveals essential functions of the homeodomain transcription factor *Shox2* in sinoatrial and pacemaking development. *Circulation* 115: 1830-1838, 2007.
- 23 Blaschke RJ, Monaghan AP, Schiller S, Schechinger B, Rao E, Padilla-Nash H, Ried T and Rappold GA: *SHOT*, a *SHOX*-related homeobox gene, is implicated in craniofacial, brain, heart, and limb development. *Proc Natl Acad Sci USA* 95: 2406-2411, 1998.
- 24 Clement-Jones M, Schiller S, Rao E, Blaschke RJ, Zuniga A, Zeller R, Robson SC, Binder G, Glass I, Strachan T, Lindsay S and Rappold GA: The short stature homeobox gene *SHOX* is involved in skeletal abnormalities in Turner syndrome. *Hum Mol Genet* 9: 695-702, 2000.
- 25 Kneip C, Schmidt B, Seegebarth A, Weickmann S, Fleischhacker M, Liebenberg V, Field JK and Dietrich D: *SHOX2* DNA methylation is a biomarker for the diagnosis of lung cancer in plasma. *J Thorac Oncol* 6: 1632-1638, 2011.
- 26 Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
- 27 R Core Team: R: A language and environment for statistical computing. 2013 (cited Sep. 2013); Available from: <http://www.R-project.org>.
- 28 Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC and Muller M: pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics* 12: 77, 2011.
- 29 Schaarschmidt F. bdpv: Inference and design for predictive values in binary diagnostic tests. 2012 (cited Sep. 2013); Available from: <http://CRAN.R-project.org/package=bdpv>.
- 30 Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, Lijmer JG, Moher D, Rennie D and de Vet HC: Standards for Reporting of Diagnostic Accuracy. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. *BMJ* 326: 41-44, 2003.
- 31 Heller G, Babinsky VN, Ziegler B, Weinzierl M, Noll C, Altenberger C, Mullauer L, Dekan G, Grin Y, Lang G, End-Pfutzenreuter A, Steiner I, Zehetmayer S, Dome B, Arns BM, Fong KM, Wright CM, Yang IA, Klepetko W, Posch M, Zielinski CC and Zochbauer-Muller S: Genome-wide CpG island methylation analyses in non-small cell lung cancer patients. *Carcinogenesis* 34: 513-521, 2013.
- 32 Brock MV, Hooker CM, Yung R, Guo M, Han Y, Ames SE, Chang D, Yang SC, Mason D, Sussman M, Baylin SB and Herman JG: Can we improve the cytologic examination of malignant pleural effusions using molecular analysis? *Ann Thorac Surg* 80: 1241-1247, 2005.

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