Quescin sulfhydryl oxidase 1 (QSOX1) secreted by lung cancer cells promotes cancer metastasis

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Abstract: As lung cancer shows the highest mortality in cancer related death, serum biomarkers are demanded for the lung cancer diagnosis and its treatment. To discover lung cancer protein biomarkers, secreted proteins from primary cultured lung cancer and adjacent normal tissues from patients were subjected to LC/MS-MS proteomic analysis. Quescin sulfhydryl oxidase (QSOX1) was selected as a biomarker candidate from the proteins enriched in the secretion of lung cancer cells. QSOX1 levels were higher in 82% (51 of 62 tissues) of lung cancer tissues compared to adjacent normal tissues. Importantly, QSOX1 serum levels were significantly higher in cancer patients (p<0.05, AUC=0.89), when measured by multiple reaction monitoring (MRM). Higher levels of QSOX1 are also uniquely detected in lung cancer tissues among several other solid cancers by immunohistochemistry. QSOX1 knock-downed Lewis lung cancer (LLC) cells was less viable from oxidative stress and had reduced migration and invasion. In addition, LLC mouse models with QSOX1 knock-down also proved that QSOX1 functions in promoting cancer metastasis. In conclusion, QSOX1 might be a lung cancer tissue-derived biomarker and involved in the promotion of lung cancers, and thus can be a therapeutic target for lung cancers.

Keywords: keyword 1; lung cancer 2; biomarker 3; proteomics 4; QSOX1 5; secretome

1. Introduction

Lung cancer has been the leading cause of cancer related death in worldwide. Compared to other type of cancers, lung cancer still shows the low survival rate. According to the recent 2018 cancer statistics, lung cancer shows little increase in 5-year survival rate among all types of cancers along with pancreatic and stomach cancer [1,2]. This is largely due to the low diagnostic rate of lung cancer in the early stages. Advances of imaging technology have also been improved to detect smaller lesions than before. However, for economic burden, exposure to radiation and still high-rate of false-positive rate, other supportive types of diagnosis methods are still required in lung cancer diagnosis.

Protein biomarkers have been known to represent better the status of body than other types of biomarker molecules such as DNA. Along with advances of proteomics, protein biomarker discovery and their clinical applications has been actively studied and proposed to be a clinically valuable study by many groups [3]. Several protein biomarkers for lung cancer are developed.
including cytokeratin-19 fragments (CYFRA 21-1) [4,5], carcinoembryonic antigen (CEA) [6], neuron-specific enolase (NSE) [7], and cancer antigen-125 (CA-125)[8]. However, the challenges in biomarker discovery and long time consuming process leading to clinical application hurdle the development of biomarkers in clinical uses [9].

Lung cancer biomarker development is facing the similar problems like other cancer biomarkers. First, blood derived protein biomarkers have tendency to show cross reactivity to other types of cancers. Second, to select final biomarkers to be developed to clinical applications, large scale validation should be preceded [10]. Third, monoclonal antibodies are essential to develop immunoassay based diagnostics to be used in the clinics. Due to these hurdles, although, hundreds of biomarkers have been discovered and reported by many research groups, few are in the clinical use at present.

Secretome refers all set of molecules secreted by a living cell, a tissue or organism through any mechanisms. Secreted proteins have been proposed as a new source of biomarker discovery [11,12]. Secretome could be obtained from several different sources. In the broad range, serum or plasma is also a kind of secretome. For secretome in more proximal location, tissue effusions such as ascites or pleural effusions might contain abundant secretome. The most proximal secretome to cancers are the one collected from cancer cell cultures. Conditioned media of cells have been suggested as a complementary source of biomarker discovery [13]. It is expected that biomarker discovery from more proximal fluid could reveal more disease and cell-type specific biomarkers.

In this study, to discover lung cancer specific protein biomarkers, proteomic analysis of secreted proteins from primary cultured lung cancer cells was conducted. Selected biomarker candidate QSOX1 has been further verified in tissues and elevation of QSOX1 in the serum has been validated by MRM analysis. The role of QSOX1 in cancer progression was also assessed by knock-down of QSOX1 in LLC cells in vitro and in vivo. The results indicate that elevated QSOX1 might be a lung cancer selective biomarker and be involved in the metastasis or progression of lung cancers.

2. Results

2.1. Strategy for Lung cancer biomarker discovery from the secretome of primary lung cancer cells

To discover lung cancer selective proteins, secretome-based lung cancer biomarker discovery strategy has been applied(Figure1A). For the collection of secreted proteins from the tissues of lung cancer and adjacent normal tissues, cells from the tissues freshly acquired from the patients on surgery were separated and cultured. Normal and cancerous epithelial cells were not isolated separately but cultured together with stroma-like cells to mimic cell-to-cell interactions in in vivo environments. Cells isolated from the adjacent normal tissues showed typical cobble stone like morphology of epithelium and cells from lung cancer tissues were irregular and mesenchymal- or fibroblast-like morphology (Figure 1B).
Figure 1. MS/MS analysis of secreted proteins and GO analysis of the identified proteins. (A) Schematic diagram shows experimental process from primary culture of lung cancer tissues, secretome enrichment, and LC-MS/MS analysis of secretome. (B) From the lung cancer tissues and its adjacent corresponding normal tissues, cells were isolated by collagenase treatment. Isolated cells from cancer tissues and corresponding normal tissues show different cellular morphology. (C) Secreted proteins enriched from conditioned media of primary cultured cells of two different lung cancer patients were separated by 1D-SDS-PAGE. Separated proteins were divided into 25 bands and subjected to in-gel digestion. Tryptic peptides were subjected to LC-ESI-MS/MS analysis. MS analysis discovered 107 common proteins detected in both MS analytical sets, and 68 and 60 proteins detected only in one of set. Proteins identified in both experimental sets were subjected further GO analysis. (D) Proteins identified were analyzed by their cellular component, (E) biological process, and (F) molecular functions.

2.2. LC-ESI-MS/MS analysis of secretome and biomarker candidate discovery

Lung cancer tissues and their corresponding normal tissues of two lung cancer patients, who went operation (#20100622 and #20100719), were used for cell culture establishment. Same amount of secreted proteins enriched from the conditioned media of the normal and lung cancer-derived cells were separated by 1D-SDS-PAGE. To identify biomarker candidate proteins, gels were sliced into 25 bands. In-gel tryptic digested peptides were analyzed by LC-ESI-MS/MS. From the first and second MS analysis, 175 and 167 proteins were identified each and 107 proteins were appeared in common (Figure 1C). Identified proteins were analyzed by their cellular component, biological process and molecular functions of each protein (Figure 1D–F). As expected from several previous reports, the proteins of the conditioned media appeared to include many intracellular protein contaminants (approximately 25% of the identified proteins) from the apoptotic cell debris due to the serum deprived culture condition [15,20,21]. However, cell membrane or extracellular matrix proteins also occupy about 23% of the total proteins identified.
For the selection of biomarker candidate showing significant change between adjacent normal and lung cancer, relative peptide hits were counted after normalization by total ion current (TIC) of each mass run. Protein levels were evaluated by the value of fold change calculated by relative peptide hit of lung cancer set divided by that of normal tissue set. Proteins showing fold change over 1.5 or under 0.67 in both individuals were selected. 15 proteins out of 135 showed significant increases in lung cancer compared to normal and 4 proteins showed significant decrease in vice versa (Table 1). To find secreted protein biomarker candidates from the up and down-regulated candidates, we selected candidate which was included in extracellular matrix protein in GO analysis and has signal peptide in the amino acid sequences as a secreted protein.

Table 1. Proteins identified by LC-MS/MS analysis with increase/decrease in lung cancer sample with over 1.5-fold.

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2.3. Elevation of QSOX1 in lung cancer tissues compared to adjacent normal tissues

Among the proteins with significant change, Quescin sulfhydryl oxidase 1 (QSOX1) was selected as a lung cancer tissue biomarker candidate for further validation. In mass spectrometry analysis, four and two unique peptides of QSOX1 appeared in sample #20100622 and #20100719 respectively. Spectral counts of total peptides of normal and lung cancer samples after normalization were 5.5 and 9.1, and 1.0 and 4.3, respectively in each patient (Supplementary Table 1). To verify QSOX1 levels in lung tissues, Western blot analysis of QSOX1 in lung cancer patient’s tissues was carried out. Proteins were extracted from lung cancer tissues and their corresponding normal tissues from 62 lung cancer patients (Figure 2A). The Western blot data were quantified by densitometric analysis and the graph shows that 51 patients out of 62 (82.2%) showed the increase of QSOX1 protein levels in the lung cancer tissues compared to adjacent normal tissues (Figure 2B).

![Western blot analysis of QSOX1](image)

**Figure 2.** QSOX1 proteins present at higher level in lung cancer tissues compared to corresponding adjacent normal tissues. (A) Proteins were extracted from lung cancer tissues and their corresponding tissues from 62 lung cancer patients. For each sample, 2.5 μg of proteins were subjected to western blot analysis. (B) Graphs of densitometry analysis data showed 51 out of 62 (about 82.2%) had the increase in QSOX1 protein expression compared to adjacent normal.

To validate the QSOX1 in lung cancers, mouse model carrying Lewis lung carcinoma cell (LLC) was established. Mice were divided into 4 groups and LLC cells or PBS were intravenously injected and sacrificed at two different time points; 10 days and 35 days (Supplementary Figure 1A). Proteins were extracted from lungs without nodules and adjacent normal lung tissues of mice with lung cancer, lung cancer nodules and metastasized nodules. QSOX1 expression was tested on each sample. In accordance with human cases, lung cancer tissues from the mice also showed elevated QSOX1 level. In addition, metastasized nodules in other organs showed higher QSOX1 protein
levels (Supplementary Figure 1B). These results also suggest that QSOX1 might be involved in the metastasis of lung cancers.

2.4. Increased expression of QSOX1 preferentially in lung cancer tissues

From the results of Western blot verification in lung cancer tissues, it is confirmed that QSOX1 is up-regulated in lung cancer tissues. To further prove QSOX1 levels in tissues, immunohistochemical analysis was conducted on tissue microarray (TMA) slides of various cancer tissues with corresponding normal tissues. From the results of immunohistochemical analysis, high levels of QSOX1 protein was detected in lung cancer cells. Regardless of histological types; adenocarcinoma or squamous lung carcinoma, QSOX1 over-expression in lung cancer tissues was confirmed (Figure 3A, B and C). However, no significant increase of QSOX1 was detected in pheochromocytoma of adrenal gland, colon adenocarcinoma, large cell lymphoma of ileum, renal cell carcinoma, and hepatocellular carcinoma (Figure 3D-H). From the results, it is presumed that QSOX1 increase in lung cancer is not the cancer common response but, to some extent, lung cancer unique.
Figure 3. Immunohistochemical staining of QSOX1 in various cancer tissues and their corresponding normal tissues. QSOX1 protein expressions in lung cancer tissues were confirmed by immunohistochemistry on tissue microarray slides. Column 1 is the slides stained without antibody for corresponding normal tissues of column 2 (negative control); Column 2, stained with anti-QSOX1 antibody for each organ; Column 3, stained without antibody (negative control) for column 4; Column 4, stained with anti-QSOX1 antibody on cancer tissues for each organ. Column 5 is HE staining of column 4 at next serial section. (A, B) Lung adenocarcinoma, (C) Squamous cell lung cancer, (D) Pheochromocytoma in adrenal gland, (E) Adenocarcinoma in colon, (F) Large cell lymphoma in ileum, (G) Renal cell carcinoma, and (H) Hepatocellular carcinoma.

2.5. Validation of QSOX1 in the sera of lung cancer patients by MRM analysis

So far, we have shown that lung cancer tissues have higher levels of QSOX1 proteins of which levels were somewhat lung cancer unique among other cancers tested. To confirm whether the increase of
QSOX1 in lung cancer tissues can also be detected in the sera of lung cancer patients, MRM protocol for the QSOX1 measurement was developed. Due to the low concentration of QSOX1 in the serum, MRM has been adopted to measure QSOX1 in the sera depleted with two most-abundant albumin and IgG. Among several QSOX1 peptides recommended by Skyline program, VGSPNAAVLWLWSSHNR was finally selected based on the results of standard MRM of few samples. The m/z of precursor ions, transitions and collision energy for the selected QSOX1 peptide were optimized (Supplementary Table 2). Detailed area measurement and quantitation procedures are described in Supplement Information.

Samples of 20 healthy controls, and 40 lung cancer patients; including 20 lung adenocarcinoma and 20 squamous lung cancer patients were subjected to the MRM analysis. Three mass runs were conducted serially for each sample. Representative peak of a healthy control and a lung cancer of each peptide are shown in Figure 4A. Higher transition peak with larger peak area has been detected in lung cancer samples compared to healthy control for both peptides. There was statistically significant difference between healthy controls and lung cancer groups (Figure 4B). The diagnostic ability has been validated by Receiver Operating Characteristic (ROC) curves. Area Under curve (AUC) values of QSOX1 peptide was 0.8925 (Figure 4C). The QSOX1 peptide turned out to have high diagnostic value for lung cancer diagnosis from healthy individuals in serum.

**Figure 4.** QSOX1 proteins were higher in lung cancer patients’ sera compared to healthy controls. (A) Representative transition peak pattern of QSOX1 peptide, VGSPNAAVLWLWSSHNR, in healthy controls and lung cancer patients. (B) Box plot shows the normalized measured area of QSOX1 peptide in 20 healthy individuals, 20 adenocarcinoma patients (AdenoCA), and 20 squamous lung cancer patients (SQLC). (C) ROC for 60 individuals is plotted and area under curve (AUC) was 0.8925(* denotes P <0.05, compared to healthy individual samples in the analysis of significant variance)
2.6. QSOX1 knock-down decreases lung cancer metastasis

The LLC lung cancer mouse model experiment showed the high expression of QSOX1 in metastasized lung cancers. From this result, we hypothesized that the QSOX1 is involved in the lung cancer metastasis. First, to check whether the QSOX1 affect cancer initiation by promoting cell proliferation, cell proliferation of control cells and QSOX1 knock-downed cells was compared. There was not much difference between these two cells in proliferation rates (Figure 5A). Second, we tested whether QSOX1 could help cancer cells survive in oxidative stress. The viability of LLC cells with knock-downed QSOX1 by shRNA was more sensitive to oxidative stress conditions by H2O2 treatment and thus significantly decreased in knock-downed cells (Figure 5B). Based on this result and the reports of Shi et al [22], it can be deduced that QSOX1 secreted from cancer cells might protect cancer cells from apoptosis in tumor mass in oxidative stress condition occurred as the tumor volume grows larger. It is of note that QSOX1 secreted from fibroblast functions in regulating extracellular matrix [23]. To test ECM modulating functions of QSOX1 secreted by lung cancer cells, LLC cells were subjected to migration and invasion assays. In migration assay, the QSOX1 knock-downed LLC cells showed less migration rate than control cells (Figure 5C). Using Boyden chamber coated with basement membrane Matrigel, cell invasion assay showed that QSOX1 knock-downed cells had greatly decreased invasiveness than control cells (Figure 5D).

![Figure 5](https://example.com/figure5.png)

Figure 5. QSOX1 expression in lung cancer cells promotes cancer progression by anti-apoptotic, pro-migration and pro-invasion functions. (A) Cell proliferation of LLC transfected with shRNA control (shCont) or QSOX1 shRNA (shQSOX1) was measured by MTT assay. (B) Cell survival from oxidative stress was measured by MTT assays in various H2O2 concentration conditions. (C) Using Boyden chamber, cell migration assay was carried out and cells at the lower chamber was stained and counted. (D) Using Boyden chamber with basement membrane matrigel coated, cell invasion assay was carried out and cells at the lower chamber was stained and counted.

To confirm the QSOX1 contribution to the lung cancer metastasis in vivo, invasiveness of QSOX1 knock-downed cells was evaluated in LLC mouse model. Mice were grouped into four; mice injected intravenously with either PBS, wild type LLC cells (LLC WT), LLC with control shRNA (LLC
shCont), and LLC with shQSOX1 (LLC shQSOX1) (Figure 6A). Mice of group 2,3 and 4 had lung cancer nodules 35 days after injection and the surface nodule number was counted. The nodule numbers were not much significantly different between LLC shCont and LLC shQSOX1 groups (Figure 6B and C). However, the nodule sizes of the shQSOX1 group were significantly smaller than the nodules of the LLC shCont group (Figure 6D). When the area of nodules was measured, the tumors in QSOX1 knock-downed groups were significantly smaller than those in shCont group (Figure 6E). From these results, it can be also inferred that QSOX1 might not be involved in cancer initiation but contribute to the cancer progression such as tumor invasion and metastasis.

**Figure 6.** QSOX1 expression in lung cancer cells promotes cancer progression in vivo. (A) Mice of four groups were intravenously injected with PBS, wild type LLC, LLC with shControl and LLC with shQSOX1, respectively. (B) All of the mice injected with LLC cells (Group 2-4) had lung cancer nodules on the lung with different severity. (C) The number of surface nodules was counted and showed no significant difference between group 3 and 4. (D) Slides of the lungs were stained with H&E. (E) All nodule areas were calculated and combined for each mouse and showed significant decrease in the shQSOX1 (group 4) compared to shCont (group 3). (*indicates P <0.05 in the analysis of significant variance)

### 3. Discussion

In this study, we identified lung cancer secreted protein QSOX1 as a lung cancer selective biomarker candidate by a proteomics approach and validated the increase in tissues and serum of lung cancer patients. From the immunohistochemical analysis and LLC mouse model, the increase of QSOX1 in
The involvement of QSOX1 in cancer progression has been validated in vitro and in vivo experiments with QSOX1 knock-downed LLC lung cancer cell line.

3.1 QSOX1, lung cancer tissue selective biomarker

Along with development of proteomics technology, protein biomarkers for the lung cancer diagnosis have been reported. Many researches have been conducted to identify numerous relevant proteins in various diseases including cancers by mass spectrometry-based clinical proteomics. Although advances in mass spectrometric technologies made it possible to identify over 50,000 unique peptides covering almost 5,000 proteins, there is still limitation to uncover unique proteins.

Cancers are also considered a chronic inflammatory disease and show similar inflammatory responses [24-26]. For this reason, several acute phase proteins have been reported as lung cancer biomarkers: serum amyloid A (SAA) [27], haptoglobin (Hp) beta chain [28] and complement 9 (C9) [18]. The elevation of acute-phase proteins in the body fluid is suspected to be resulted from the secretion of the major acute phase protein-secreting organ liver by the inflammatory signals released by lung cancers. For this reason, serum biomarkers are likely to show cross-reactivity to other types of solid cancers, which could show similar systemic response.

Study of secretome from cancer cells has been suggested as an alternative source of biomarker discovery for the tissue selective biomarkers. Several studies of lung cancer secretome have been conducted. Lung cancer cell lines have been subjected to the study, but not many studies have been conducted on primary lung cancer cells or organ cultures [29]. Studies on secretome of about 15 different lung cancer cell lines have been reported. According to the studies, new proteins have been reported, which were not been discovered in the serum as lung cancer biomarkers. These biomarker candidates include Cathepsin-D [30], L-lactate dehydrogenase B chain (LDHB) [31], translationally controlled tumor protein (TCTP) [32], triosephosphateisomerase (TPI) [32], dihydrodiol dehydrogenase (DDH) [33] etc. However, QSOX1 has never been discovered in the secretome analysis of lung cancer cell lines.

The main limitation of cell secretome study is that 2-dimensional cell culture cannot fully mimic complex cancer microenvironments [13]. To partially overcome the concerns, stromal supporting cells isolated from the tissues were cultured together without any sorting. Therefore, the cancer cells were maintained with other different heterogeneous cells that include different types of immune cells infiltrated in the cancer tissues and fibroblasts. From the Western blot analysis of tissue protein, we confirmed the increase of QSOX1 and immunohistochemical analysis of tissue microarray revealed that QSOX1 expression was increased in the lung cancer tissues.

3.2 Establishment of MRM for the measurement of QSOX1 in sera

Multiple Reaction Monitoring (MRM) or Single Reaction Monitoring (SRM) is mass-spectrometry-based protein/peptide quantification methods [34,35]. MRM depends on mass spectrometry and its current limit of quantitation (LOQ) is at the low attomole level [36,37]. The strength of this technique is that it is one of the methods best suited to the validation of hundreds of protein biomarkers in large number of samples. Compared to the conventional clinical method, such as ELISA, MRM shows highly correlated data and even better results when the target shows saturated results in ELISA [38].
To detect serum proteins at lower concentrations, stable isotope standards with capture by anti-peptide antibodies (SISCAPA)\[36,39\] or depletion of abundant proteins have been combined in MRM. To overcome the wide range of serum proteins, abundant protein depletion in the sample preparation step can precede MRM analysis. This step increases the chance of detecting proteins at lower concentrations in the sera. Following the depletion of abundant Albumin and IgG in the serum, optimal conditions of QSOX1 MRM analysis methodology have been established. The QSOX1 was detected in the sera of lung cancer patients although the level is very low. Lung cancer patients showed higher levels of serum QSOX1 compared to healthy individuals. The AUC curve for differential diagnostics was close to 0.9. This implies that QSOX1 increase in the sera can be a selective lung cancer biomarker than blood-born biomarkers of which most shows high cross-reactivity to other types of solid cancers.

3.3 QSOX1 plays role in cancer progression and metastasis

QSOX1 has been first reported to be expressed in human lung fibroblast. QSOX1 expression was increased as the cells reach to high confluence and suggested that it might have role in induction and maintenance of quiescent state of cells \[40\]. Since the report of QSOX1 protein, enzymological studies of the protein have been conducted \[41-43\]. It is reported that QSOX1 catalyze the generation of disulfides by reducing oxygen to hydrogen peroxide as Erv family. The tissue expressions of QSOX1 have also been reported in several studies. In breast cancer, it is reported that high expression of QSOX1 leads to the reduction of tumorigenesis and correlated to the prognosis of the patients \[44\]. In pancreatic cancer, association between QSOX1 and cancer metastasis has been studied \[45\]. However, QSOX1 has never been reported in lung cancer and their biological function in cancer progression has never been thoroughly studied. From a recent study, it has been suggested that QSOX1 secreted from the fibroblast modulates extracellular matrix by enzymatic incorporation of laminin \[23\]. According to the previous studies and our results of the knock-down experiment of the QSOX1 in vitro, it is suggested that QSOX1 secreted from the cancer cells promotes cancer metastasis by modulating extracellular matrix as it does in fibroblasts. In accordance with previous studies, our results showed that QSOX1 can promote cancer progression by promoting cancer metastasis in vitro and in vivo. It is also reported that inhibition of enzymatic activity of QSOX1 suppressed invasion of pancreatic and renal cancer cell lines \[46\] and QSOX1 inhibited the autophagic reflux \[47\]. Inferred from these two published data and our results, it is suggested that QSOX1 might be a therapeutic target for lung cancer.

4. Materials and Methods

4.1 Human tissues and serum samples

Tissue and serum samples were obtained from patients at Seoul National University Bundang Hospital, (IRB# B-1201/143-003) and Samsung Medical Center (IRB No. 2008-06-007-005). Informed consent was obtained from all donors. Serum was separated from whole blood by centrifugation within 4hrs after collection and was stored at -70°C until use. Tissues were obtained during the surgery and for the primary culture delivered in the RPMI media with antibiotics. Tissues for the storage were rapidly cooled by liquid nitrogen. The information of patients who provided the samples used in the experiments is summarized in Supplementary Table 3.

4.2 Cell culture

4.2.1 Primary culture of lung tissues
Primary culture of lung pneumocytes was carried out by modified protocols of previously reported study [14]. Lung tissues freshly collected in DMEM media were chopped by blades and treated with collagenase Type I (0.5mg/ml) in 37°C, shaking incubator for 1.5 hrs. Tissue clumps were removed by filtration with mesh and isolated cells were plated on culture dishes and cultured in media containing 10% fetal bovine serum for 4 days. Then, dead or unattached cells were washed away. After one passage subculture for the amplification, same number of cells was seeded on the plate for the conditioned media collection. Normal and cancerous epithelial cells were not isolated separately but cultured together with stromal cell types to mimic cell-to-cell interactions in in vivo environments.

4.2.2 Cell line culture

Lewis lung carcinoma (LLC) cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM/High glucose) (Hyclone) containing 10% fetal bovine serum (Hyclone) and 1% antibiotic-antimycotics (GIBCO, Grand Island, NY).

4.3 Secretome collection and protein precipitation

Same number of cells derived from lung cancer tissues and adjacent normal tissues were plated on 100mm dishes. After 24hrs culture in 10% FBS supplemented DMEM, when reached 60-70% of cell confluency, media was changed to serum free media to collect conditioned media (CM) containing secretome. To remove contaminants from FBS, cells were washed with pre-warmed PBS for four times and pre-warmed serum free media for two times before media change. After 24hrs, CM was harvested [15,16]. To remove cell debris, collected media was centrifuged at 2000 rpm, 10min. and supernatant was further filtered by 0.2μm pore filter. Secreted proteins were enriched from filtrated conditioned media by TCA (Trichloroacetic acid) purification method. In brief, 1volume of 100% (w/v) TCA (Sigma Aldrich) was added to 4 volumes of media, then incubated 4hrs at 4°C and centrifuged at 13000 rpm, for 30min. Supernatant was removed, leaving protein pellet intact. Pellet was washed with cold acetone and then re-dissolved in RIPA buffer (Thermo Scientific) with protease inhibitor cocktail (Roche). Protein concentration was measured by Bradford assay (Bio-Rad).

4.4 Proteomic analysis of secretome protein

Following one-dimensional gel electrophoresis and Coomassie staining, gels were excised and subjected to in-gel trypsin digestion as previously described [17]. Briefly, after destaining in 75 mM ammonium bicarbonate/40% ethanol (1:1), gels were subjected to reducing (5 mM DTT in 25 mM ammonium bicarbonate) and alkylating (55 mM iodoacetamide) conditions each at room temperature for 30 min. After dehydration with ACN, 20 μg/ml sequencing grade trypsin (Roche Applied Science) containing 25 mM ammonium bicarbonate was added and the slice was incubated at 37°C overnight. Tryptic peptides were eluted with 0.1% formic acid.

LC-MS/MS analysis was performed using Thermo Finnigan ProteomeX work station LTQ linear IT MS (Thermo Electron, San Jose, CA, USA) equipped with NSI source (San Jose, CA). Analysis conditions for mass spectrometry were the same as previously reported [18]. MS/MS data were searched based on the IPI human protein database (version 3.29) using the SEQUEST algorithm (Thermo Electron). Scaffold ver. 01_07_00 was used to validate MS/MS based peptides and protein identification.

4.5 Knock-down of QSOX1 and in vitro analysis

4.5.1 Knock-down of QSOX1

shControl vector and shQSOX1 in retroviral vectors were purchased from Origene, USA. LLC cells were transfected with shRNA control vector and shQSOX1 by electroporation, condition of 1100 mv, 20 pulses and 20 msec. Transfection was first confirmed by GFP images. Cells transfected
with plasmids were selected with puromycin 4.5μg/ml and then survived cells were maintained in 0.1 μg/ml puromycin containing media.

4.5.2 Proliferation Assay

Same number of cells were seeded on 96-well plates and cell number was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay every 24 hrs.

4.5.3 Measurement of anti-apoptotic ability in hypoxic stress

2.0×10⁴ number of LLC cells was seeded on 24-well plates. After 24 h, the medium was replaced by the complete medium containing H₂O₂ at the final indicated concentrations. After 8hrs of treatment, toxicity and cell survival was assessed by MTT assays.

4.5.4 Migration and Invasion Assay

Cell migration and invasion assays were performed in 24-well trans-well plates (8-μm pore size, Corning, USA). Matrigel (BD Bioscience, USA) was diluted to 1 mg/ml with serum- free culture medium and applied on the insert in the upper chambers of the multiwell for invasion assay plate. 1.0×10⁶ cells in 200 μL of serum-free culture medium were seeded in the upper chambers of the wells. To induce chemotaxis of cells, 800 μL of 10% FBS medium was added to the lower chambers. After incubation for 24hrs at 37°C and 5% CO₂, the membrane inserts were removed from the plate, and non- invading cells were removed from the upper surface of the membrane. Migrated or invaded cells were stained with 0.1% crystal violet for 20min and washed with water. The invading cells were counted in at least 5random fields using a microscope. The migrated cells were counted in 5 random fields by cell confluence measuring program in JuLI FL microscope (NanoEntek, Korea).

4.6 In vivo metastasis assay in mouse model

Seven to eight weeks old C57BL/6 male mice were purchased from OrientBio (Daejeon, Korea). To establish LLC mouse model, mice were i.v. injected with 1.5×10⁶ LLC cells through tail vein. Mice were scarified ten days and 32 days after tail vein injection and lung, liver, and serum were collected for evaluation. All animal experiment was approved by institutional animal care and use committee (IACUC) of Seoul National University. (SNU-131213-3)

4.7 Statistical analysis

For statistical analysis, a p-value calculator for the student t-test and ANOVA was used. The results of each group were subjected to statistical analysis to assess differences compared to the control group. A p-value of less than 0.05 was considered to be significant.

5. Conclusions

Here, our results proved the secretome analysis of primary cancer and its stromal cells could find tissue selective biomarkers. We found that QSOX1 can be a serum biomarker as detected by MRM and used in lung cancer diagnosis. In addition, from its functions in promoting cancer metastasis, it might be further studied as a therapeutic target of lung cancer.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Patients’ clinical information, Table S2: Sequence and number of QSOX1 peptide identified by LC-MS/MS analysis, Table
S3: Q1/Q2 Transitions for MRM experiments, Figure S1: Verification of QSOX1 increase in LLC mouse lung cancer model.

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