

TECHNICAL NOTE

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# Proteomic and bioinformatic analysis of recurrent anaplastic oligodendroglioma

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## Abstract

**Background:** Anaplastic oligodendroglioma (AO) is a type of glioma that is believed to originate from oligodendrocytes in the brain or from glial precursor cells. Recurrence of AO reduces the overall survival rate of patients and causes meningeal or even systemic spread/metastasis more frequently than other types of gliomas. We performed proteomic analysis of recurrent AO tumors to identify the proteins significantly expressed in recurrent AO and to understand biological characteristics of recurrent AO.

**Findings:** Using human brain tissues, we identified 401 proteins that were significantly expressed in recurrent AO. Through bioinformatic analysis, we determined that the majority of the identified proteins are involved in anti-apoptotic pathway and cell proliferation. In addition, our findings suggest that epidermal growth factor (EGF) signaling may be responsible for the development of recurrent AO.

**Conclusions:** These results will aid researchers in understanding the pathology of recurrent AO and identifying the therapeutic targets for the treatment of recurrent AO.

## Findings

The goal of proteomics is a comprehensive, quantitative description of protein expression and its changes under the influence of biological perturbations such as disease or drug treatment (Anderson & Anderson 1998; Blackstock & Weir 1999). Proteins are vital parts of living organisms, as they are the main components of the physiological metabolic pathways of cells (James 1997). Recently, a number of proteomic studies have focused on the metabolic and signaling pathways associated with the formation and progression of tumors. The information obtained from proteomic analysis can be used to identify biomarkers or therapeutic targets of tumors (Alterovitz et al. 2008).

Anaplastic oligodendroglioma [AO, World Health Organization (WHO) grade III] is a type of glioma that is believed to originate from oligodendrocytes in the brain or from glial precursor cells. AO occurs primarily in adults (9.4% of all primary brain tumors), but also

sometimes occurs in children (4% of all primary brain tumors) (Allison et al. 1997). AO is distinguished from other brain tumors by a unique constellation of molecular genetic alterations, including the coincident loss of chromosomal arms 1p and 19q in 50–70% of tumors (Gregory et al. 1998). AO also has a genetic profile similar to that of other primary gliomas, such as amplification of epidermal growth factor receptor (EGFR) expression, 10q loss, p16 deletion, and phosphatase and tensin homolog (PTEN) mutation without 1p loss or tumor protein 53 (TP53) mutation (Anthony et al. 2003). The standard treatment for AO is surgical resection followed by radiation and chemotherapy with procarbazine, lomustine, and vincristine (PCV) (Kyritsis et al. 1993; Allison et al. 1997; Cairncross et al. 1992; Cairncross & Macdonald 1988; Cairncross et al. 1998; Glass et al. 1992; Kleinberg et al. 1993; Mason et al. 1996). Despite high response rates ( $\geq 70\%$ ) to treatment, the median survival period is relatively short (Glass et al. 1992). Accumulation of diverse genetic and epigenetic alterations that occur due to interventions for treatment of the tumor may cause extensive changes in the expression of genes involved in oncogenesis leading to treatment-related effects such as aggressive transformation (Kleinberg et al. 1993).

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Despite advances in the treatment and understanding of primary AO, recurrent AO is significantly less well understood. Although a recent study reported the microRNA expression profile in patients with recurrent AO (Kim et al. 2011), the biological characteristics of recurrent AO have never been studied. In this study, we conducted proteomic analysis of recurrent AO to examine protein profiles in recurrent AO and identify biological and molecular characteristics of recurrent AO.

## Materials and methods

### Patient samples and protein extraction

Institutional Review Board of the Catholic University of Korea approved of this study. The three specimens were obtained from patients with recurrent AO treated with total excision and adjuvant radiotherapy with total dose of 55.8 Gray (Additional file 1: Table S1) (Kim et al. 2011). The specimens were separated during surgery at the Seoul St. Mary's Hospital and stored in liquid nitrogen at  $-80^{\circ}\text{C}$ .

Protein extraction was conducted previously described (Cao & Liang 2012) with minor modification. The specimens is added to RIPA buffer (89900, Pierce Biotechnology, IL, USA) containing protease inhibitor cocktail (78410, Pierce), and sonication. Centrifuge 14,000 rpm,  $4^{\circ}\text{C}$ , 20 min, and store supernatant at  $-80^{\circ}\text{C}$ . After, the supernatant was used for SDS-PAGE and LC-MS/MS.

### SDS-PAGE and in-gel digestion with trypsin

Protein samples were separated by 12% SDS-PAGE (mini-PROTEAN, BIO-RAD). A 100  $\mu\text{g}$  of protein sample was applied to each lane, and the gels were stained with Coomassie Brilliant Blue R-250. In-gel digestion was conducted in accordance with the previously described method (Kim et al. 2006). Gels were fractionated into 10 parts according to molecular weight. Each part was digested with trypsin (1.2  $\mu\text{g}$ ) for 16 hours at  $37^{\circ}\text{C}$  after reduction and alkylation of cysteines of the proteins. Digested peptides were extracted by extraction solution (50 mM ammonium bicarbonate, 50% acetonitrile, and 5% trifluoroacetic acid). Digested peptides were resolved in 10  $\mu\text{l}$  of sample solution containing 0.02% formic acid and 0.5% acetic acid, and stored at  $-80^{\circ}\text{C}$  until required.

### LC-MS/MS analysis and protein identification

The peptide samples (5  $\mu\text{l}$ ) were concentrated on a Easy-column<sup>TM</sup> (L 2cm, ID 100 $\mu\text{m}$ , 120 $\text{\AA}$ , C18-A1) trapping column (PROXEON, Denmark). Peptides were eluted from the column and directed onto a Easy-column<sup>TM</sup> (L 10cm, ID 75 $\mu\text{m}$ , 120 $\text{\AA}$ , C18-A2) reversephase column (PROXEON, Denmark) at a flow rate of 200  $\text{nl}/\text{min}$ . Peptides were eluted by a gradient of 0~65% acetonitrile for 120 min. All MS and MS/MS spectra in the LTQ-

Velos ESI ion trap mass spectrometer (Thermo Scientific, USA) were acquired in a data-dependent mode. Each full MS ( $m/z$  range of 300 to 2,000) scan was followed by seven MS/MS scans of the most abundant precursor ions in the MS spectrum with dynamic exclusion enabled. For protein identification, MS/MS spectra were searched by MASCOT (Matrix science, [www.matrixscience.com](http://www.matrixscience.com)). The proteome sequence database of IPI human ver. 3.82 (<http://www.ebi.ac.uk/IPI>) was used as the database. Mass tolerance of parent ion and fragment ion was 1.5 Da and 1.3 Da, respectively. Carbamidomethylation of cysteine and oxidation of methionine were considered in MS/MS analysis as variable modifications of tryptic peptides. Mascot score was recalculated by percolator function in the mascot for increasing sensitivity of correct identification and false discovery ratios (FDR) of the search results was adjusted to below 2%.

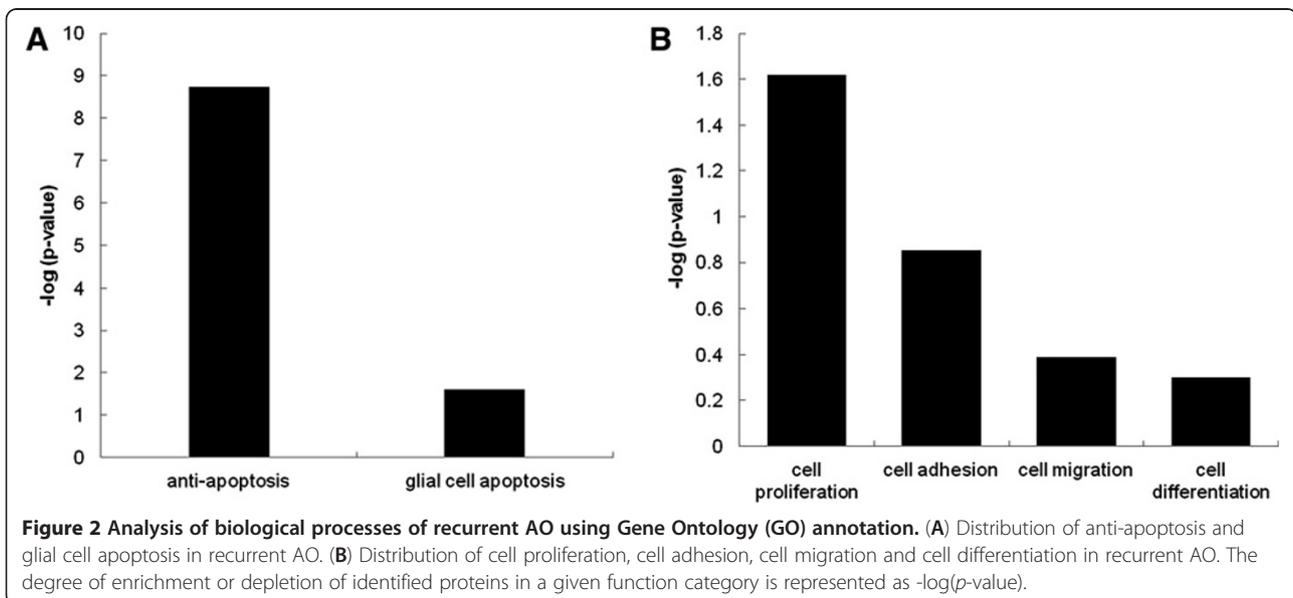
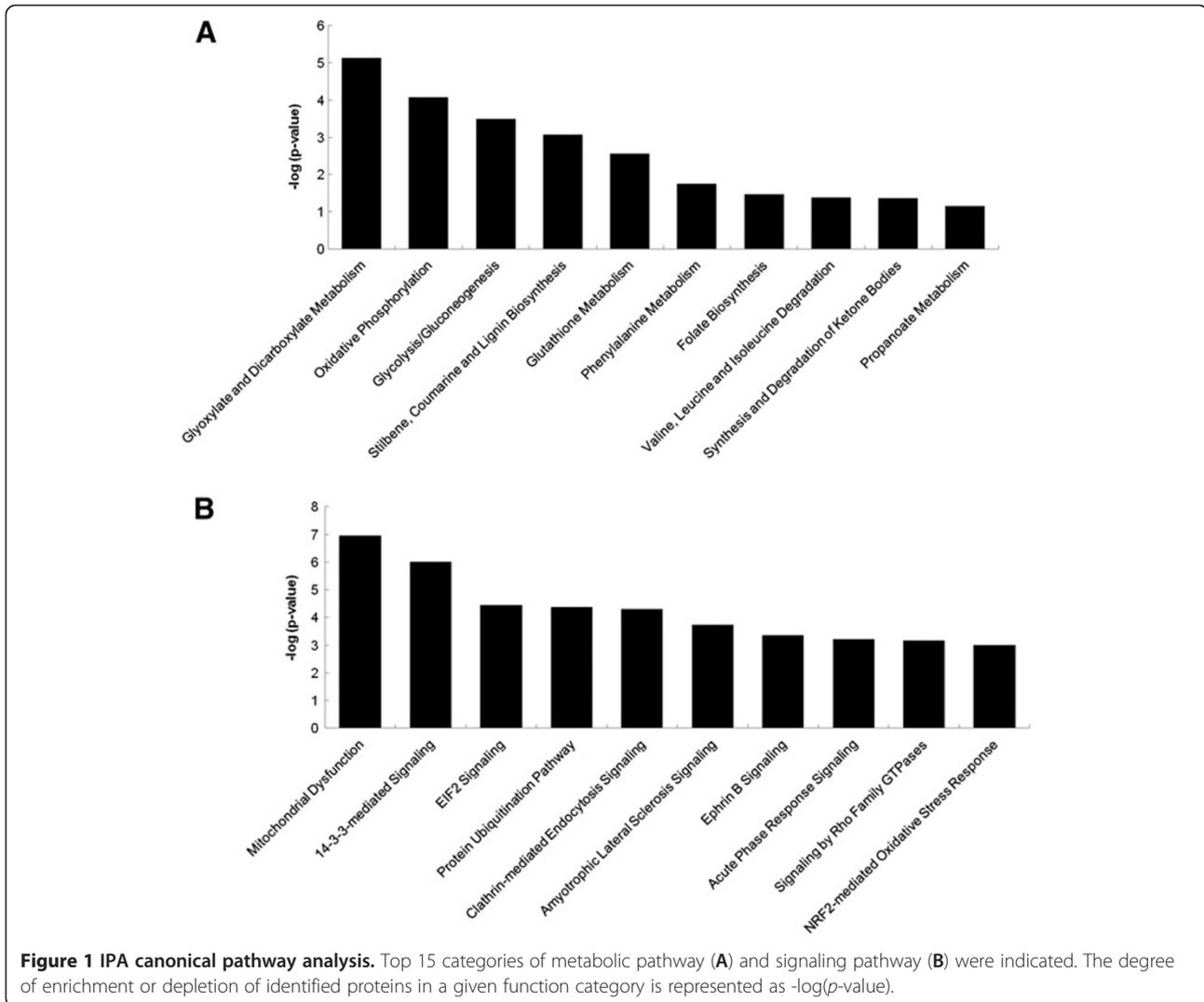
### Bioinformatic analysis

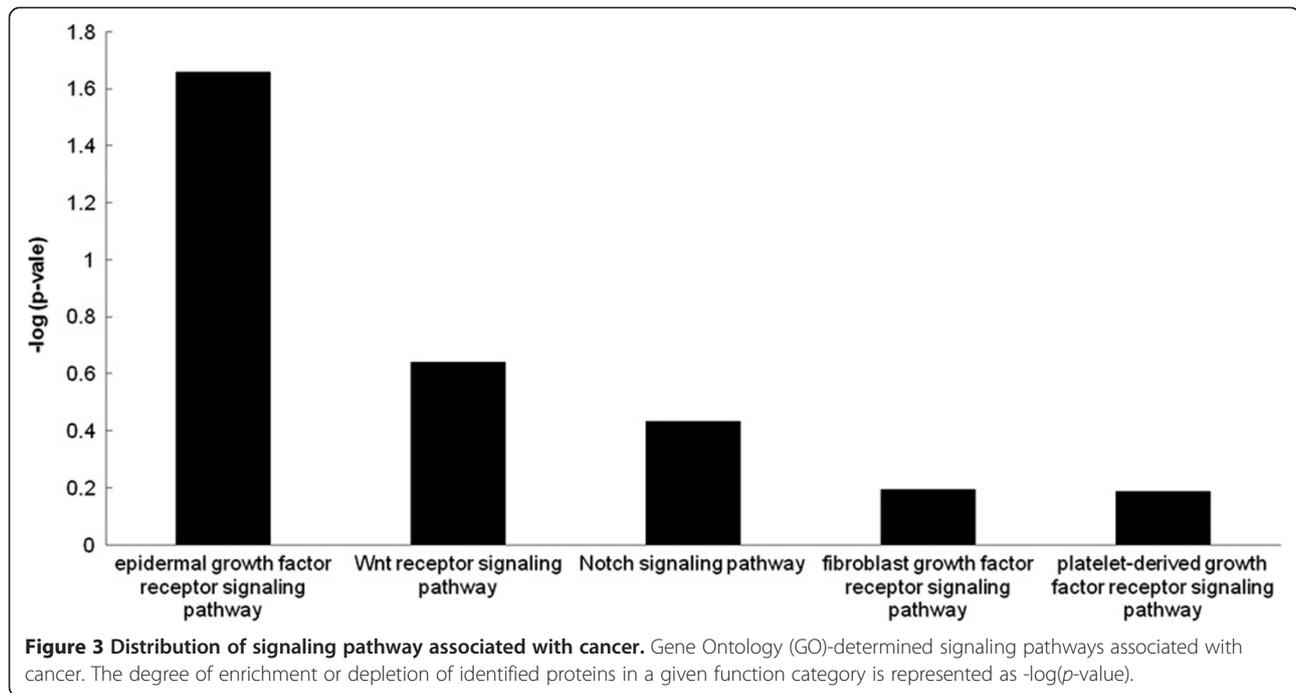
Using Ingenuity Pathway Analysis (IPA) tool, all of the identified proteins in recurrent AO were subjected analysis of indicated metabolic and signaling pathway. The identified proteins in recurrent AO were subjected to query global protein network analysis and direct/indirect interactions on proteins that are involved in pathways associated with epidermal growth factor receptor (EGFR). For functional enrichment analysis of Gene Ontology (GO) categories, GOfact online tool (<http://61.50.138.118/gofact>) was used. *p*-value of Fisher's exact test determines the probability that the association between the proteins in the dataset and the biological function/pathways explained by chance alone.

## Results and discussion

### Proteomic analysis of recurrent AO

First, we performed proteomics analysis using tumor sample from patient with recurrent AO who were treated with postoperative chemoradiotherapy. From LC-MS/MS, 401 proteins were shown significantly to express in recurrent AO (Additional file 2: Table S2) and then we analyzed the 401 identified proteins using the Ingenuity Pathway Analysis (IPA) tool (<http://www.ingenuity.com>). The results revealed the majority of the metabolically associated proteins that was involved in biosynthesis of carbohydrates, such as glyoxylate and dicarboxylate metabolism, oxidative phosphorylation and glycolysis/gluconeogenesis (Figure 1A). With respect to the signaling pathways active in recurrent AO, the identified proteins were associated with pathways responsible for cell growth (Figure 1B). These findings



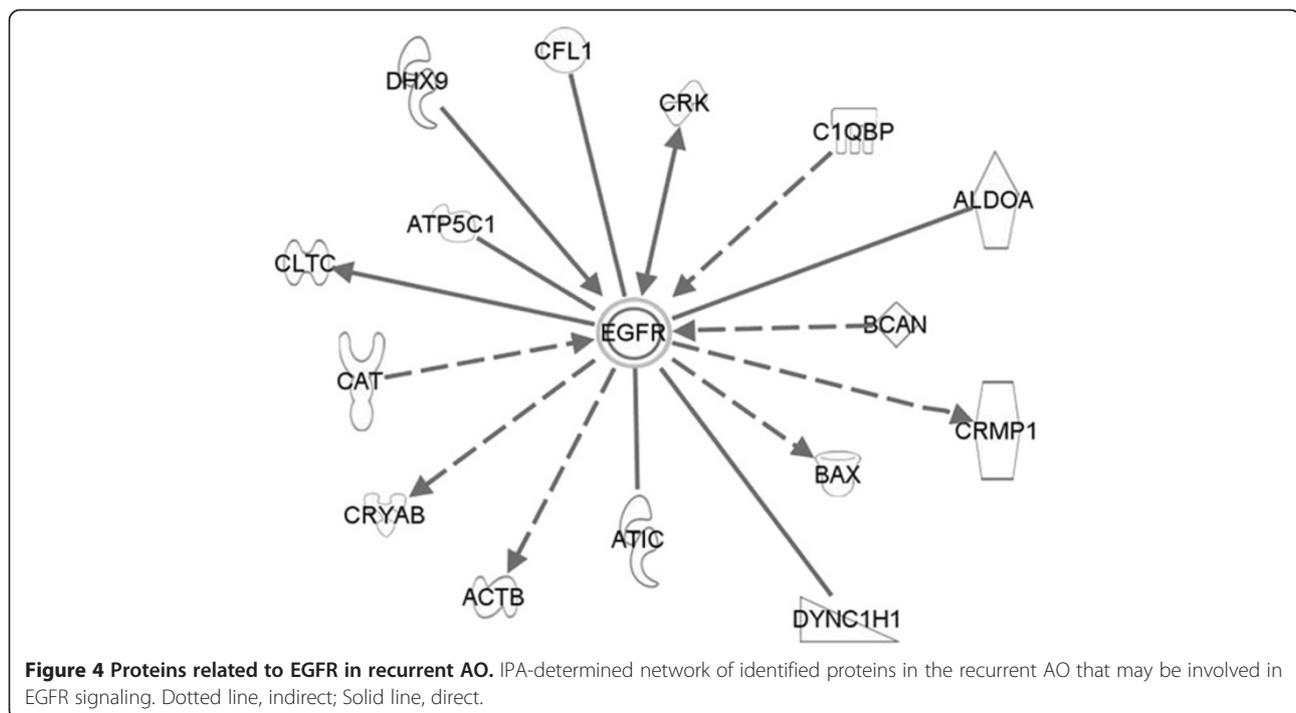


indicate that recurrent AO may be in the biological state of actively growth.

#### Biological process of recurrent AO

To confirm the biological state of actively growth and discover the specific cause of recurrent AO, Gene Ontology

(GO) annotation analysis was performed. As shown in Figure 2A, the results indicated that the identified proteins were connected to anti-apoptotic functions, while only a few were associated with glial cell apoptosis. This may indicate why recurrent AO is resistant to chemoradiotherapy. Anti-apoptotic effects often contribute to cancer cell survival and



chemoresistance in cancer cells (Williams et al. 2005). The 18 proteins that are involved in anti-apoptosis pathways are listed in Additional file 1: Table S3.

Multiple biological processes, including cell proliferation, adhesion, and migration, have been shown to be involved in tumor development (Lee 1992). Based on our analyses, the 14 proteins appear to be linked to cell proliferation processes (Figure 2B). Fourteen of these proteins are presented in Additional file 1: Table S4. Tumor cell is related to cell adhesion and motility. Cell motility is necessary to move within tissues during invasion and metastasis by their own motility (Ymazaki et al. 2005; Zang et al. 2006). The 11 proteins and 9 proteins appear to be linked to cell adhesion and migration, respectively (Additional file 1: Table S5 and Table S6). These findings indicate that the expression of proteins related to anti-apoptotic and cell proliferation pathways are closely related to the development of recurrent AO. Understanding the functions of these proteins in recurrent AO would contribute to the development of therapeutics for the prevention or treatment of this disease.

#### Signaling pathways associated with tumor development in recurrent AO

Recent studies claimed that growth factor signaling is involved in tumorigenesis and the development of malignancy (Lee 2001). EGF signaling is well known to be involved in the autonomous growth of cancer cells (Normanno et al. 2001), and FGF signaling plays a pivotal role in cancer development (Cao et al. 2011). Wnt and Notch signaling are also associated with tumor development (Allenspach et al. 2002; Paul 2000). Therefore, we investigate whether the identified proteins are involved in various signaling pathways. The results showed that EGF signaling is highly associated with recurrent AO compared to other signaling pathways (Figure 3). This is in agreement with a previous report showing that amplification of EGFR, which permits evasion of cancer cell death, has been observed in cases of recurrent AO that develop resistance to treatment (Paul 2000). Additionally, Wnt and Notch signaling were also associated with recurrent AO. Further studies regarding the role of Wnt and Notch signaling during the development of AO recurrence will be necessary.

#### Proteins related to EGFR in recurrent AO

Based on the fact that EGF signaling is highly related to recurrent AO (Figure 3) and activation of EGFR is responsible for the resistance of recurrent AO to treatment, novel regulators or effectors that mediate EGFR signaling in recurrent AO were sought. EGFR signaling plays roles in cell proliferation, angiogenesis, and inhibition of apoptosis processes that are essential for cancer

development (Normanno et al. 2001). By additional bioinformatics analysis of the identified proteins identified using IPA tool, we indeed found that 15 proteins are closely associated with EGFR (Figure 4 and Additional file 1: Table S7), implying that these proteins may play an important role in the development of recurrent AO.

#### Conclusion

In this study, we identified proteins significantly expressed in recurrent AO. Extensive bioinformatics analysis revealed that these proteins are involved in biological processes and signaling pathways that are associated with the development of recurrent AO. Cells in recurrent AO are highly resistant to apoptosis and actively proliferating. Analysis of signaling pathway enrichment also showed that EGF signaling is active in recurrent AO. This is the first analysis to identify and characterize the proteins associated with recurrent AO. Further studies, investigating the role of these identified proteins in recurrent AO will contribute to a more thorough understanding of the molecular mechanisms that mediate the development of this disease.

#### Additional files

**Additional file 1: Supplementary tables.**

**Additional file 2: Table S2.**

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

YH and ECP designed experiments, analyzed data, and wrote the manuscript. EYS performed proteomic analysis and SOK carried out bioinformatic analysis. YTO and BOC shared evaluation of patients and sampling. GK and GHK supervised the study. All authors read and approved the final manuscript.

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