J Chin Med Assoc

ORIGINAL ARTICLE

Redefined complement C3c structures have significant increase in the plasma of ovarian cancer patients

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Abstract

Background: Ovarian cancer (OC) is the third most common gynecological cancer. Effective biomarkers are required for OC as in the case of other cancers. Therefore, here we explored whether plasma proteolytic products could serve as potential biomarkers.

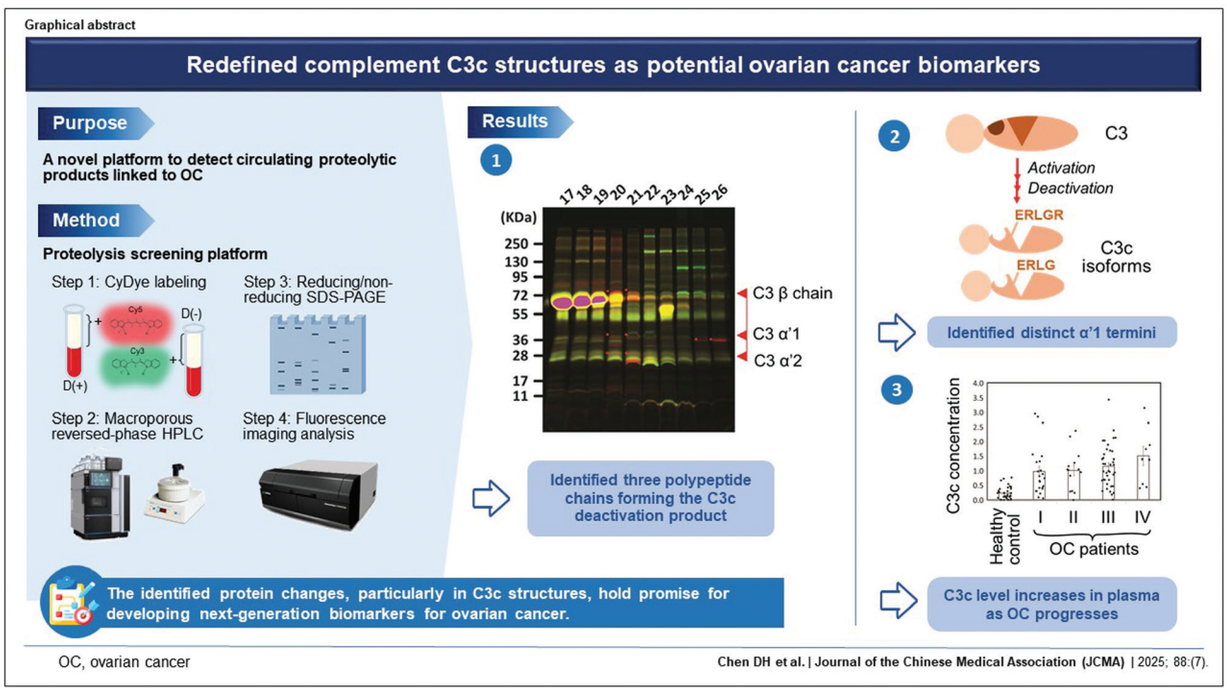
Methods: We devised a platform that incorporates CyDye labeling, macroporous reversed-phase liquid chromatography, reducing/nonreducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAG), and fluorescence imaging. Paired preoperative and postoperative plasma samples from four patients were used to screen for possible proteolytic changes. For identified difference proteins, liquid chromatography-tandem mass spectrometry was used to analyze the protein digests using various proteases. Plasma samples from 33 healthy controls and 85 patients with OC were examined using an enzyme-linked immunosorbent assay.

Results: Our analyses revealed that the circulating complement C3 derivative was present only in the diseased state. This 145-kDa species, under nonreducing conditions, could split into 72-, 39-, and 29-kDa fragments upon reduction, reminiscent of the C3c structure. While confirming the C3c identity, mass spectrometric analyses showed multiple C-terminal ends in the C3c α'1 fragment, which were utilized differently among patients with OC. Various ends were also observed in serum samples prepared using different complement activators, thus redefining C3c as a mixture of multiple molecular entities. Enzyme-linked immunosorbent assay (ELISA) targeting only canonical C3c demonstrated a strong correlation between increased plasma levels and the occurrence and progression of OC.

Conclusion: Our findings suggest that plasma proteolysis during complement deactivation is explicitly involved in ovarian tumorigenesis and the associated protein changes may aid in developing next-generation cancer biomarkers.

Keywords: Biomarkers; Complement C3; Mass spectrometry; Ovarian neoplasms; Proteolysis

Graphical abstract



Lay Summary: Ovarian cancer is a serious disease that affects many women worldwide, but early detection can significantly improve survival rates. In this study, researchers analyzed blood samples to identify biomarkers that could signal the presence of ovarian cancer. They developed an innovative method to compare blood samples taken from patients before and after surgery and identified a unique protein form known as complement C3c. This specific form was found exclusively in the blood of women with ovarian cancer. Complement C3c consists of distinct fragments, and its presence may reflect underlying mechanisms of cancer progression. Using a straightforward blood test, the scientists confirmed that elevated levels of this protein variant were strongly associated with both the presence and the stage of ovarian cancer. This breakthrough could pave the way for improved diagnostic blood tests, enabling earlier detection, faster intervention, and ultimately offering women a better chance at successful treatment and recovery.

1. INTRODUCTION

Ovarian cancer (OC) is the third most common type of gynecological cancer and is characterized by poor prognosis and high mortality rates.[1,2] The treatment of OC is often delayed owing to the lack of specific symptoms and the shortage of high-sensitivity tests for early diagnosis. This highlights the urgent need to identify effective biomarkers. Despite decades of research, progress in discovering early-stage OC biomarkers has been limited. The well-known biomarker, CA-125, has been used as an individual indicator. However, it has demonstrated unsatisfactory sensitivity and specificity for OC surveillance.[3] The human epididymis protein 4 (HE4), approved by the FDA in 2008 as a monitoring biomarker, has shown promise in detecting recurrence, however, it does not perform better than CA-125 in early detection.[4]

Complement proteins can be activated via three distinct pathways: classical, lectin, and alternative. These pathways converge to activate the complement component 3 (C3). Upon the initiation of the complement responses, C3 undergoes an initial processing step that involves the removal of the C3a fragment, resulting in the conversion of C3 to C3b. Activated C3b species can bind to the surface of transformed cells through the activation of the thioester moiety Cys-1010-Gln-1013 in the thioester-containing domain (TED).[5] For the deactivation of C3b, factors I (FI) and H (FH) can convert the bound C3b into iC3b, which is followed by the release of a small C3f piece. Another cleavage occurs between the C3dg and C3c α'1 fragments, releasing C3c from the C3dg anchored at the cell surface.[6] This intricate process highlights the complexity of the complement system and its role in immune responses.

We developed an analytical platform to identify the protein species resulting from proteolysis associated with OC. Preoperative and postoperative plasma samples from the same patient were analyzed using a sequence of processes, including Cy3/Cy5 dye labeling, macroporous reversed-phase (mRP) liquid chromatography,[7] reducing/nonreducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and fluorescent image analyses. Different species were characterized using mass spectrometry. This platform has enabled the discovery of a complement protein that exhibits increased proteolysis in the diseased state.

2. METHODS

2.1. Ethics statement

The study adhered to the Declaration of Helsinki and was approved by the Institutional Review Board of the National Taiwan University Hospital (protocol code 202105112RIFA). Informed consent was obtained from all the participants.

2.2. Blood sampling

Blood samples were collected from the forearm vein and placed into vacutainer tubes with or without ethylenediaminetetraacetic acid (EDTA). Serum and plasma were prepared, aliquoted, and stored at –80°C.

2.3. Fluorescent dye labeling and mRP liquid chromatography

Minimal labeling with CyDye fluorochromes (Cy5, Cy3) was performed on pooled plasma samples collected preoperatively and postoperatively from four patients, following previously reported procedures.[8] The mRP chromatography was performed on CyDye-labeled proteins according to the manufacturer's instructions, except that no reducing agent was added before sample loading.[7] The fractions were collected at a speed of 1 tube per min and stored at –80°C for later use.

2.4. Serum C3 activation

Serum C3 was activated as previously described.[9] C3 activators, heat-aggregated immunoglobulin G (HAIgG), and zymosan A (Sigma-Aldrich, St. Louis, MO) were used at a final concentration of 1 or 10 mg/mL, respectively. To obtain HAIgG, human IgG solution (Sigma-Aldrich) was aggregated at 63°C for 15 minutes and then cooled on crushed ice until IgG precipitated. C3 activation was carried out on an automatic mixer at 37°C for 4 hours. Excess zymosan A was removed by centrifugation at 3220 × g for 30 minutes at 20°C. The resulting supernatants were further centrifuged at 20 800 × g for 30 minutes at 4°C. The final supernatant was aliquoted and stored at –80°C.

2.5. SDS-PAGE and western blot analyses

Reducing and nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis analyses were performed using a standard protocol. For the analysis of CyDye-labeled proteins, fluorescent images were collected using an FLA-5100 scanner (Fujifilm, Tokyo, Japan) with settings of 450/580 nm for Cy3 and 400/670 nm for Cy5. MultiGauge (version 3.0; Fujifilm) was used for image presentation. Electrophoretic transfer was performed according to standard protocols. For immunoblotting, rabbit antibodies to C3c α'2 or C3c α'1 were diluted 1:2000 with 1% bovine serum albumin (BSA). Horseradish peroxidase-conjugated antibodies against rabbit IgG (Jackson ImmunoResearch) were diluted 1:10 000 in 1% BSA. The membranes were visualized using ImageQuant LAS 4000 (GE Fujifilm).

2.6. In-gel digestion and mass spectrometric analyses

Gels subjected to mass spectrometric analysis were first stained with Coomassie blue dye. The bands of interest were excised

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Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

Author contributions: Dr. Dong-Hao Chen and Dr. Yi-Wen Chang contributed equally to this work.

Journal of Chinese Medical Association. (2025): 88: 568-576.

Received December 7, 2024; accepted March 3, 2025.

doi: 10.1097/JCMA.0000000000001249

from the gel and the proteins were digested in-gel using trypsin, Lys-C, or Asp-N (Roche). The digested peptides were desalted with Ziptip U-C18 (Millipore) and analyzed by mass spectrometry with Vanquish Neo UHPLC system connected to Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA). Raw data were formatted using ProteoWizard MSConvert (v3.0.23006) and processed using CRUX against the human protein database from UniProt, downloaded in March 2023.[10,11] Mass spectrometry proteomic data are available in the ProteomeXchange Consortium via the PRIDE repository (dataset identifier PXD057890).[12]

2.7. Enzyme-linked immunosorbent assay

C3c concentrations were measured using a FineTest EH2737 ELISA kit (Wuhan Fine Biotech, China) according to the manufacturer's instructions. Standards and samples were analyzed in duplicate and absorbance was measured at 450 nm using a TECAN Infinite M200 PRO plate reader. Two-tailed *t* tests were used to compare results between the healthy controls (HC) and stage I groups.

3. RESULTS

3.1. Derivatives of C3 were detected specifically in the plasma of patients with OC

To identify the circulating proteolytic structures that could serve as OC indicators, we used four pairs of plasma samples collected before and after treatment. We pooled preoperative samples and labeled the proteins with Lys-reactive Cy5 dye. Similarly, the pooled postoperative samples were chemically tagged with Cy3 dye. Following the combination of these samples, we resolved the proteins using mRP liquid chromatography under nonreducing conditions, resulting in a series of eluted fractions. By using reducing and nonreducing SDS-PAGE, we identified protein species that exhibited significant changes through fluorescence image analyses (Fig. 1A). We observed several proteins that showed none-to-all changes in patients with OC.

We were particularly interested in a group of protein bands in the 20 to 21-minute mRP fractions, as those were identified as C3 derivatives through mass spectrometry-based analyses (Figs. 2A, 3A). Under reducing conditions, the three species, each with comparable intensity, had molecular masses of 72, 39, and 29 kDa, respectively (Fig. 1B). Tryptic peptides of the 72-kDa species were mapped to the sequence segment corresponding to the 645-residue β chain. The other two species apparently originated from two distinct regions of the α chain (Figs. 2A, 3A). Using nonreducing SDS-PAGE, one 145-kDa species was found in the same morph fraction from patients with OC (Fig. 1C), suggesting that this C3 derivative consists of three C3 fragments linked with thiol-sensitive bonds. Clearly, this 145-kDa C3 derivative is different from the commonly seen plasma C3, or C3 α-β heterodimer, based solely on molecular size (Supplementary Fig. S1, https://links.lww.com/JCMA/A331).

We used the same platform technology to identify differential proteolysis in paired preoperative and postoperative plasma samples obtained from four patients with breast cancer. However, the overall pattern of differential proteolysis differed markedly from that observed in patients with OC (data not shown). Notably, we did not observe similar all-or-none changes, suggesting that the C3 derivatives originated from an OC-specific process.

3.2. OC-associated C3 species have structural features shared by the deactivated product C3c

To map the sequences of the 39- and 29-kDa species in the α chain, we first closely examined their tryptic peptides with mass spectrometric analyses. For the larger species, these peptides covered the segment spanning residues 1321 to 1663 (Fig. 2A), corresponding to the range of C3c α' chain fragment 2 (referred to as C3c α'2 henceforward). We also established that Ser-1321 was the true N-terminal of this C3 fragment by observing the same N-terminus in the Asp-N peptide (Fig. 2B, C). Therefore, we conclude that the 39-kDa species is C3c α'2 (Supplementary Fig. S1, https://links.lww.com/JCMA/A331). Moreover, western blot analyses revealed that this fragment was exclusively present in the four samples collected preoperatively and was not observed in their postoperative counterparts. These results were consistent with the all-or-none changes observed for paired samples in the fluorescent imaging analyses (Fig. 1).

For the 29-kDa species, we identified Ser-749 as the N-terminal residue by analyzing the tryptic digests. This residue is supposed to be the N-terminal end for the α'1 fragment of the C3c (Fig. 3A; Supplementary Fig. S1, https://links.lww.com/JCMA/A331). We found Arg-954, the canonical C-terminal end of the C3c α'1 fragment, in a tryptic peptide. From the Lys-C digest, we identified peptide 941-TVAVRTLDPERLGR-954 sharing the same end (Fig. 3B). Additionally, we discovered a slightly shorter version in the same analysis with Gly-953 at its C-terminus (Fig. 3C). As these two Lys-C peptides had comparable MS signals, we supposed that the 29-kDa polypeptide in patients with OC was a mixture of two C3c α'1 fragments, differing only by one residue (Fig. 3A and Supplementary Figure S1, https://links.lww.com/JCMA/A331).

3.3. Different C3c α'1 ends were present in the plasma C3c from most patients with OC and in the serum C3c prepared based on various activation protocols

Having observed an alternative terminus based on the analyses of pooled OC plasma, we investigated the prevalence of the Gly-953 end in C3c from another group of stage I patients. Western blotting showed that all these patients had C3c α'1-containing structures (Fig. 4). Lys-C digests were prepared for these C3c α'1 fragments and then subjected to mass spectrometric analyses, targeting 941-TVAVRTLDPERLGR-954 and 941-TVAVRTLDPERLG-953. Most of the two Lys-C peptides were triply charged, with a small fraction containing up to +2 ions. Despite being one residue longer, the former migrated earlier in the reversed-phase column, probably because of the extra Arg residue carrying an additional positive charge. In four of the five patients, the longer peptide was the predominant form, whereas the other peptide was detectable. Notably, the digest from one patient (P5) had the shorter one as the major product, with signals 4.5 times higher than those for the other (Table 1). Thus, both C3c α'1 ends, which define two C3c species, were present in all plasma samples from the five patients with OC.

Given that Arg-954 is the only C3c α'1 end reported in the literature to date, we sought to determine whether the generation of multiple C3c α'1 ends was an OC-associated process. To address this, we examined the α'1 fragment in serum C3c species prepared using two different protocols. The serum complement system was activated by incubation with either heat-aggregated immunoglobulin G (HAIgG) or zymosan A. The former is known to induce the classical pathway, whereas the latter activates the system through the lectin pathway. Although the 120-kDa C3 α chain was still abundant in the plasma of patients with OC (Fig. 4), a majority of this subunit disappeared in the treated serum and appeared to convert to C3c α'1 fragment (Fig. 4). With the robust production with either reagent, the C3c α'1 fragment was prepared and analyzed using the same mass spectrometry.

Mass spectrometric results clearly discerned the Lys-C peptides. More products with different ends were detected in both preparations (Table 1). The second-shortest peptide eluted

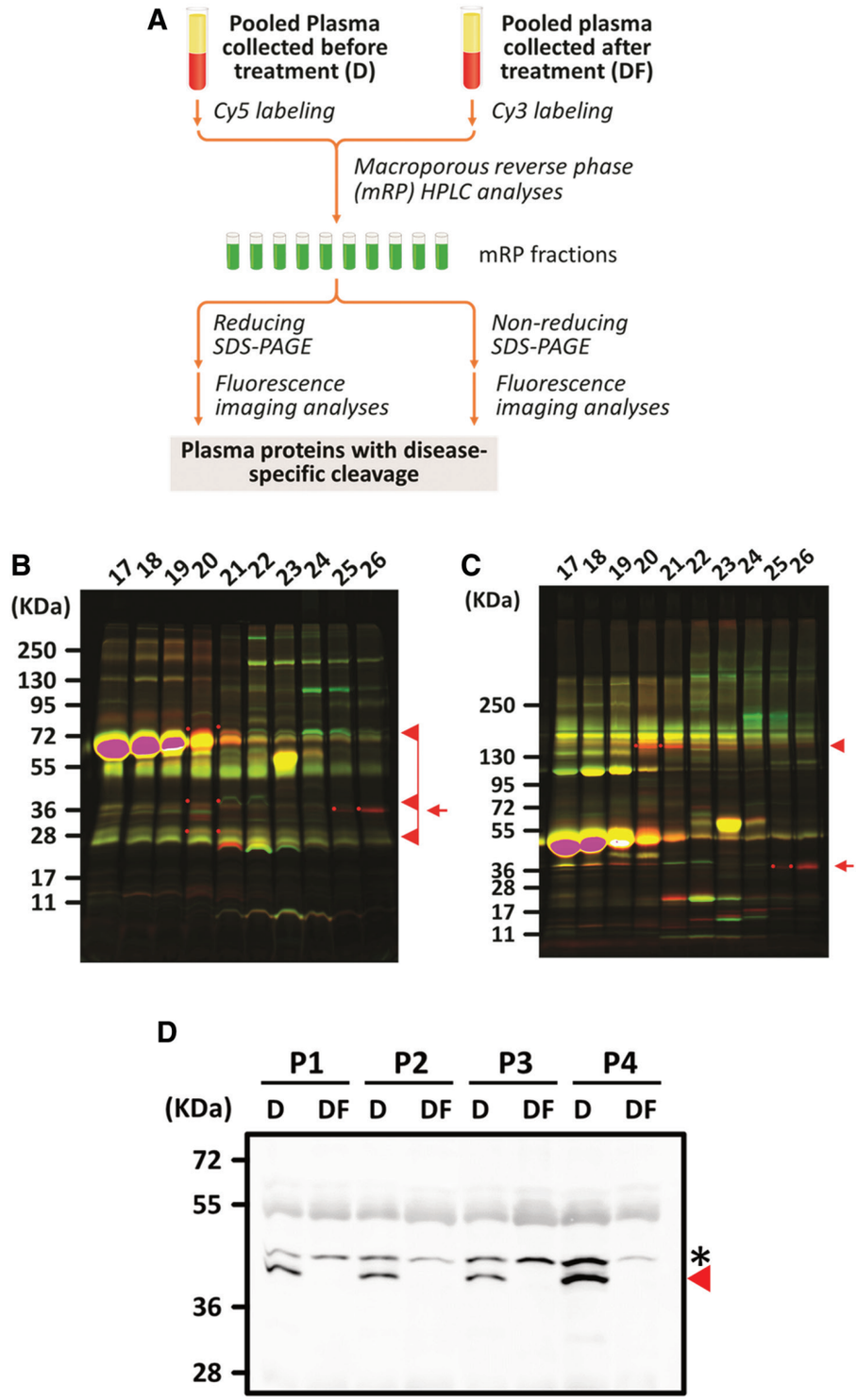

Fig. 1 Schematic and the results using mRP chromatography-based differential analyses. A, Pooled samples from the same group of patients with OC before (diseased; D) or after therapy (DF) were incubated with respective CyDye, and their proteins were analyzed successively with mRP-HPLC, SDS-PAGE, and fluorescent image analyses. B, Polypeptides in specified mRP fractions were analyzed using 10%-15% SDS-PAGE under the reducing condition. The arrowheads linked by a vertical line denote the positions of three C3c fragments and the arrow marks, where C3dg migrated. C, The same set of samples was analyzed using nonreducing SDS-PAGE. The arrowhead marks the position of the C3c conjugate; the arrow indicates the gel position of C3dg. The numbers at the top in panels B and C are the elution times of mRP fractions and those to the left are the sizes of molecular mass markers. D, Western blot analyses of the respective samples used in A with antibodies against C3c α'2 fragment. The arrowhead marks the position of the 39-kDa species, with a nonspecific band marked with the asterisk. DF = disease-free; mRP = macroporous reversed-phase; OC = ovarian cancer; SDS-PAG = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

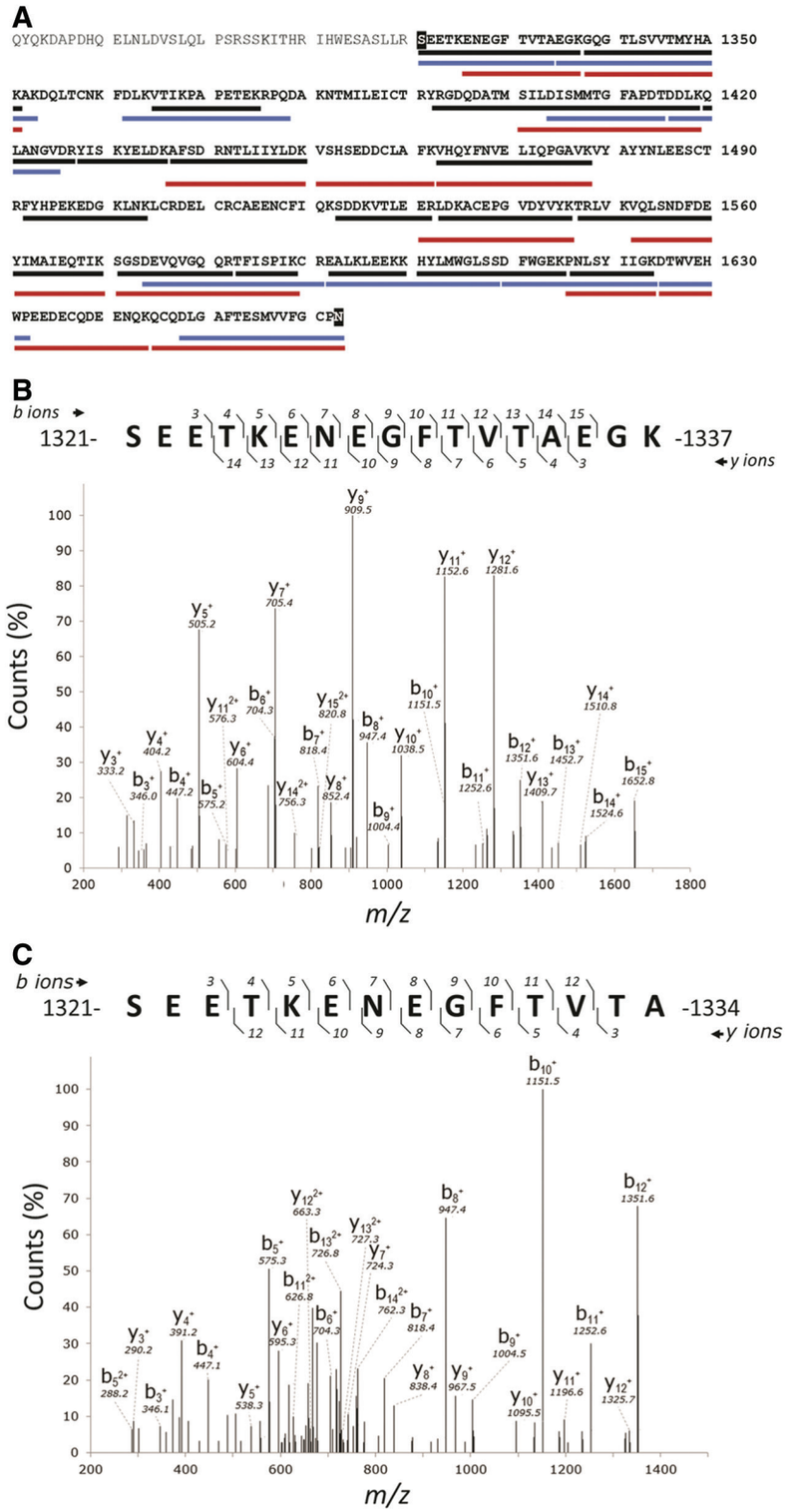

Fig. 2 Summary of mass spectrometry-based protein identification analyses of three different protease digests of the 39-KDa polypeptide. A, The black, blue, and red bars indicate the tryptic, Asp-N, and Lys-C peptides, respectively, identified in these analyses. The numbers to the right are the positions of amino acids in the nascent products of complement component C3 (primary accession: P01024). The residues highlighted by black boxes specify the determined ends for this C3 fragment. B, The tandem mass spectrum of tryptic peptide 1321-SEETKENEGFTVTAEGK-1337. The slant lines at the top of the sequence mark the detected b ions and those at the bottom are for observed y ions. C, The tandem mass spectrum of Asp-N peptide 1321-SEETKENEGFTVTA-1334. The denotation of b and y ions is the same as that shown in B.

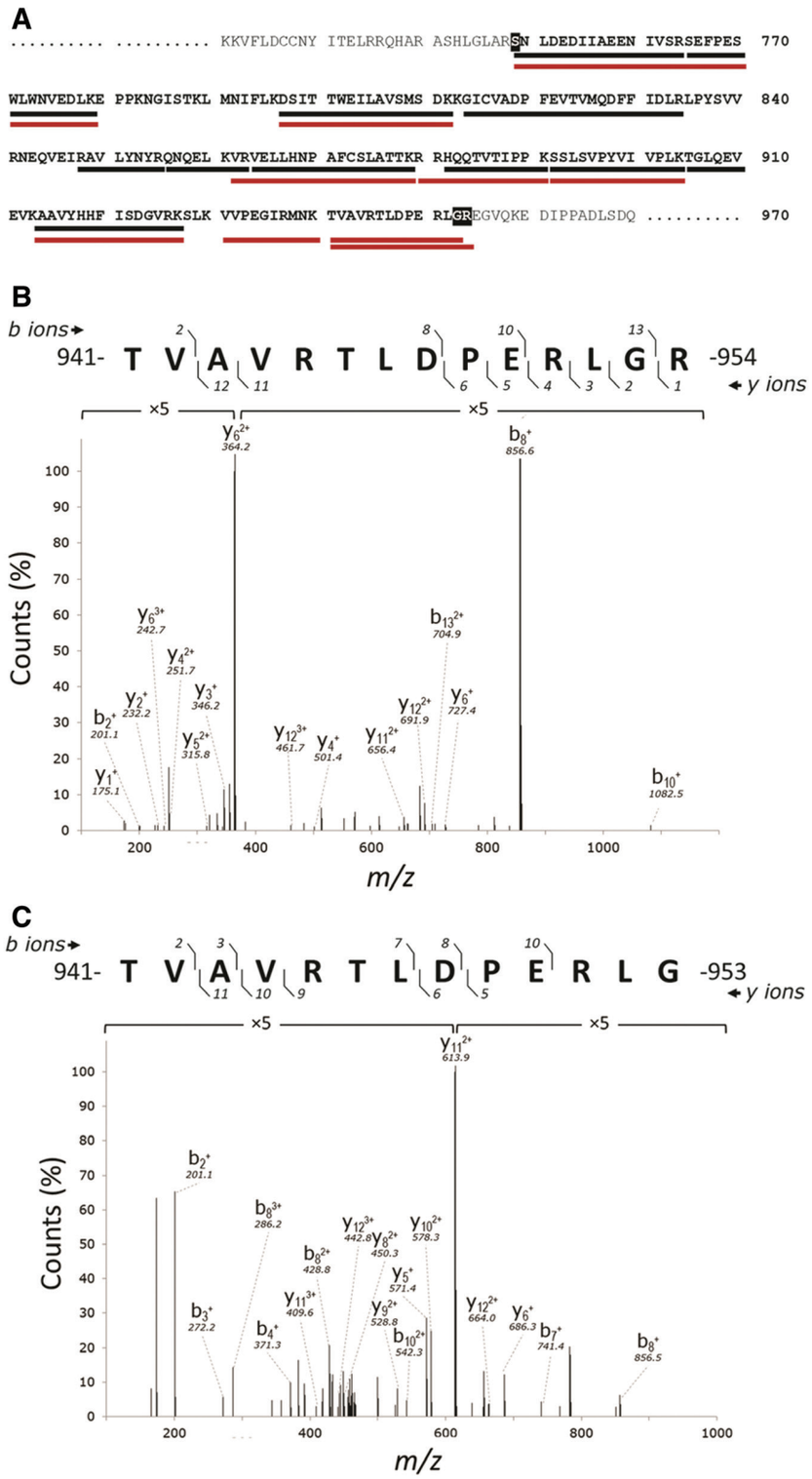

Fig. 3 Summary of mass spectrometry-based protein identification analyses of two protease digests of the 29-kDa polypeptide. A, The black and red bars underline the tryptic and Lys-C peptides identified in these analyses. The numbers to the right indicate the positions of amino acids in the complement component C3. The residues highlighted by black boxes specify the ends documented for this C3 fragment. B, The tandem mass spectrum of Lys-C peptide 941-TVAVRTLDPERLGR-954. C, The tandem mass spectrum of Lys-C peptide 941-TVAVRTLDPERLG-953. The slant lines at the top of the sequence mark the detected b ions and those at the bottom are for observed y ions.

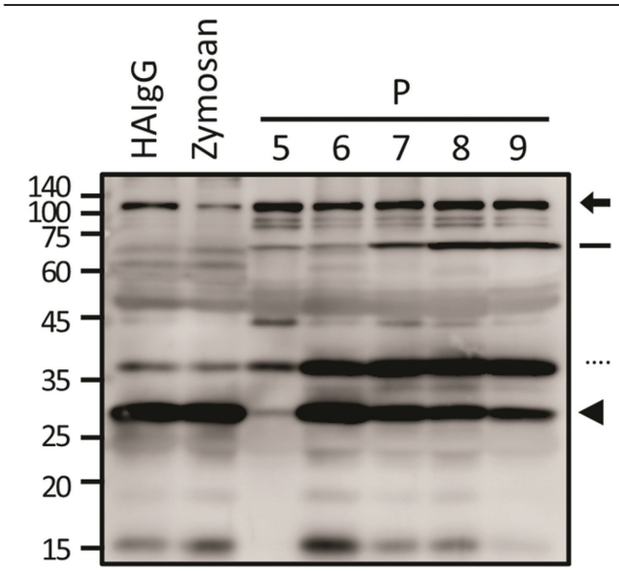

Fig. 4 Western blot analyses of C3 derivatives using antibodies against the C-terminal sequence of α'1 fragment. HAIgG and zymosan A were used as serum complement activators. Plasma samples from five stage I patients with OC were also used for mass spectrometric analyses. The arrowhead and arrow indicate the positions of the C3c α'1 fragment and the C3 α chain, respectively. The additional C3 fragments in the plasma were marked with solid or dashed lines. HAIgG = heat-aggregated immunoglobulin G; OC = ovarian cancer.

the earliest among the four and the removal of an additional Arg resulted in delayed elution, reminiscent of the migration of 941-TVAVRTLDPERLG-953 compared to that of 941-TVAVRTLDPERLGR-954. Unlike the other three Lys-C peptides, the shortest peptide was detected primarily as doubly charged cations. More importantly, the second-longest peptide had signals comparable to those of the Lys-C peptide with an Arg-954 end (Table 1). Thus, our results indicated that serum C3c species with distinct C3c α'1 ends are produced via either activation pathway. Therefore, the C3c species should be redefined as a mixture of multiple isoforms.

3.4. Enzyme-linked immunosorbent assay verified that canonical C3c concentration increased in association with the occurrence and progression of OC

The current C3c enzyme-linked immunosorbent assay (ELISA) utilizes a monoclonal antibody that targets the synthetic peptide with Arg-954 as its α'1 end.[13] As this ELISA kit is for canonical C3c, we investigated whether this C3c species increased in most patients with OC. For HC, the mean of C3c concentrations was approximately 0.2 μg/mL, with the vast majority having concentrations lower than 0.5 μg/mL. For stage I patients with OC, the average concentration was approximately 1 μg/mL, significantly higher than that of healthy subjects. The plasma concentration increased at more advanced stages, as the mean became ~1.5 μg/mL for stage IV patients (Fig. 5). Collectively, our results suggest that an increase in circulating C3c structures is associated with the occurrence and progression of OC. More samples will be analyzed using ELISA assay and the effectiveness of the C3c test in identifying patients with OC will be evaluated.

4. DISCUSSION

In this study, we used a comparative analysis based on nonreducing mRP chromatography to identify proteolytic products with a specific increase in patients with OC. In one particular mRP fraction, LC-MS/MS analyses revealed the presence of complement C3c species with a molecular mass of 145 kDa and intact disulfides that disintegrated into 72-, 39-, and 29-kDa fragments upon reduction. In addition to the canonical C-terminal Arg-954 of the serum C3c α'1 fragment, several additional C-terminal ends were present due to the removal of 1 to 4 extra residues. For most patients with OC, C3c with the α'1 Arg-954 end was the predominant species; however, some patients had the α'1 with the Gly-953 end as the major isoform. Enzyme-linked immunosorbent assay further confirmed the association between increased C3c levels and OC occurrence and progression.

4.1. Nonreducing mRP chromatography is a valuable tool for identifying plasma proteins that undergo disease-associated proteolysis

Interchain and intrachain disulfides play crucial roles in stabilizing the overall structure of circulating proteins. For instance, albumin contains 17 intrachain disulfide bonds of 35 cysteine residues. These bonds are stable in environments exposed to atmospheric oxygen, such as blood plasma. Several plasma proteins undergo proteolysis, either physiologically or pathologically, which often results in multiple fragments held together by disulfides. Processes that mediate C3 activation and C3b deactivation may serve as prime examples. In most biological experiments, reducing agents are used to prevent protein oxidation. However, these reagents should be avoided when analyzing disulfide-rich structures. Hence, we conducted mRP liquid chromatography without adding reducing agents (Fig. 1A). Once the multichain disulfide conjugates were separated into fractions, SDS-PAGE analyses were performed under reducing or nonreducing conditions. This approach facilitated the characterization of subunit structures connected by disulfide linkages, such as the OC-associated C3c.

Table 1

Signal intensities (×10^5 arbitrary units) of C-terminal Lys-C peptides of the C3c-α'1 fragment in the serum treated with either complement activator or in the plasma samples from five patients with OC

Charge		HAIgG		Zymosan A		P5		P6		P7		P8		P9	
Peptide	RT ± SD (min)	+2	+3	+2	+3	+2	+3	+2	+3	+2	+3	+2	+3	+2	+3
TVAVRTLDPERLGR	25.2 ± 1.4	2.0	240	1.1	49	0.0	1.3	2.0	98	3.0	86	2.0	140	2.0	29
TVAVRTLDPERLG	29.3 ± 2.4	7.0	190	3.9	19	0.0	5.9	1.0	9.7	1.0	9.9	2.0	20	0.0	10
TVAVRTLDPER	19.9 ± 1.1	0.0	5.0	0.2	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TVAVRTLDPE	23.2 ± 3.4	7.0	0.0	9.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

HAIgG = heat-aggregated immunoglobulin G; OC = ovarian cancer; RT = retention time.

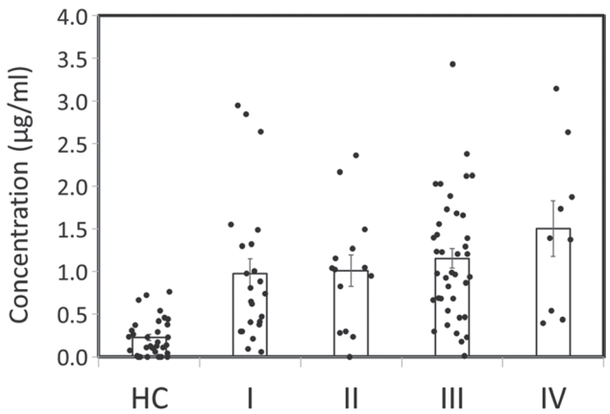

Fig. 5 ELISA analyses of plasma C3c concentrations in HC and stages I-IV patients with OC. The bars show the mean concentrations for these groups and the error bars indicate SEs of means. *p* value between the results of HC and stage I (OC) groups was <0.001 using two-tailed *t* tests. ELISA = enzyme-linked immunosorbent assay; HC = healthy controls; OC = ovarian cancer.

4.2. Additional C3c α'1 ends verified in the activated serum samples were present in the plasma C3c from patients with OC

We confirmed that the serum C3c α'1 fragment had multiple distinct C-terminal ends using serum activator. Patients with OC have a mix of C3c structures and the use of these ends may vary. To date, C3c has been known to result from the cleavage of iC3b, which is supposed to be the product of C3b deactivation. Factor I, in conjunction with factor H, is presumed to be a protease that mediates the two-step deactivation process.

The bond cleaved by FI to produce C3c α'2 is in the sequence SLLR-SE and that to produce C3c α'1 is RLGR-EG. Cutting of the latter presumably occurs following cleavage between the C3dg and C3c α'2 segments. The FI protein also acts on the C4 protein at two sites: HRGR-TL and STGR-NG. FI-mediated proteolysis is characterized by the effective processing of these motifs embedded in protein substrates, however, is not as efficient for synthetic peptides. To date, no reports of FI catalyzing faltering proteolysis or harboring carboxypeptidase activity have been reported.

Enhanced C3c formation in patients with OC is a process considered to be a marker of inflammatory conditions.[14] Thus far, no report is available on how plasma C3c elevation is associated with OC or any other cancer. Complement C3 plays a pivotal role in promoting the formation of membrane attack complex.[15] C3 is also involved in triggering other downstream proteins to elicit immune responses. Here, enhanced C3c formation likely resembles the forced termination of C3b-mediated action, consistent with the notion that C3 deactivation is a counteracting measure of antitumor immune responses.

The enzyme FI, along with its partner FH, is considered responsible for C3c conversion. High expression of FI has been demonstrated in OC tissues.[16] This over-expression is assumed to play a role in immune escape by avoiding complement attack on tumors, such as non-small cell lung carcinoma and gliomas.[17] OC cells are known to secrete FH or its functional analogs.[18] Thus, the enhanced C3c formation observed may be due to an OC-associated increase in FI- or FH-like activity.

4.3. ELISA assay targeting additional C3c α'1 ends holds the potential to improve the detection of OC and other health conditions

A commercially available C3c sandwich ELISA with an anti-terminal monoclonal antibody was developed to detect canonical C3c in the plasma. Initially devised for detecting inflammatory conditions, this kit was also shown to be a tool for detecting heart diseases. For instance, studies have shown that circulating C3c levels are paradoxically downregulated in patients with ventricular septal defects, however, can be restored through corrective surgery.[19] Patients with coronary artery disease have been found to have elevated plasma C3c levels.[13] We expect that the C3c produced under these conditions would also be a mixture of those with distinct ends. As we discovered that multiple C3c species could be produced in certain patients, the ELISA could be further improved by including antibodies that target alternative ends.

In conclusion, our study provides insights into the deactivation of complement C3 in OC, presenting opportunities for discovering new therapeutics and developing novel diagnostic tests. Future studies involving larger patient cohorts are necessary to validate these findings and explore the clinical utility of C3c as a biomarker for OC.

ACKNOWLEDGMENTS

This study was funded by the OncoLock Co. Ltd.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://links.lww.com/JCMA/A331.

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