

Bioinformatics Analysis of the Subcellular Distribution of the Human Serpin Superfamily Reveals Putative Nuclear Localization and Nuclear Export Signals, Suggesting Potential Intracellular Roles of Interest

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Article

Bioinformatics Analysis of the Subcellular Distribution of the Human Serpin Superfamily Reveals Putative Nuclear Localization and Nuclear Export Signals, Suggesting Potential Intracellular Roles of Interest

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Abstract

The serpin (serine protease inhibitor) superfamily is the largest class of protease inhibitors, involved in proteolytic cascades and mostly serving as serine/cysteine proteinase inhibitors. Serpins are involved in various biological functions: coagulation, fibrinolysis, angiogenesis, and have crucial roles in various diseases, including cardiac fibrosis, Alzheimer's, emphysema, obesity, and diabetes. Based on the subcellular distribution profile, vertebrate serpins are classified as intracellular and extracellular serpins. Clade B serpins are considered ancestral serpins, mainly found inside the cells. Clade C serpins were the first to appear extracellularly and most likely bridge intracellular and extracellular serpins. Surprisingly, few reports indicated that secretory serpins such as Plasminogen activator inhibitor-1 (PAI-1) are localized in various subcellular compartments. The intracellular localization of such serpins prompted us to investigate whether the other members of the human serpin superfamily were also inside the cells. Here, we showed for the first time that various secretory serpins from clades A, D, E, H, and I are not obligatory extracellular and were also found in different subcellular compartments, such as the nucleus and centrosome. Surprisingly, secretory serpins tend to shuttle between the nucleus and the cytoplasm as they possess either a nuclear localization signal, a nuclear export signal, or both. Intriguingly, the intracellular localization of secretory serpins is not in line with the evolution-based origin of secretory serpins, suggesting that the secretory serpins acquired additional extracellular functions during evolution, in addition to the intracellular functions. These findings will help decipher the novel intracellular functions of secretory serpins.

Keywords: Serpin; Protease; PAI-1; nuclear localization signal; nuclear export signal; centrosome

Introduction

The serpin (serine protease inhibitor) superfamily is the largest class of protease inhibitors (PIs), comprised of ~1500 members, and is found in all living organisms, i.e., from viruses and prokaryotes (bacteria and archaea) to humans [1,2]. Most of the serpins are involved in proteolytic cascades and serve as serine/cysteine proteinase inhibitors, but a few have novel functions too, such as hormone transport and blood pressure regulation [2]. Serpins are involved in various biological functions: coagulation, fibrinolysis, angiogenesis, and have crucial roles in various diseases, including cardiac fibrosis, Alzheimer's, emphysema, obesity, and diabetes [3]. They have a conserved secondary

structure consisting of three β sheets and nine α -helices and utilize an irreversible suicide-substrate mechanism of inhibition. Serpins contain an exposed reactive center loop (RCL) that imitates the protease substrate [4]. It interacts with target proteases, and the serpin and the protease are irreversibly inactivated [4]. Serpins are classified into 17 clades, of which 9 belong to vertebrate serpins (clades A-I) [5]. In another type of classification considering various biological criteria, i.e., genomic organization, amino acid sites, and indels, vertebrate serpins are further classified into six groups (V1-V6) [6]. Based on the subcellular distribution profile, vertebrate serpins are classified as intracellular and extracellular serpins. Clade B serpins are considered ancestral serpins, mainly found inside the cells [7]. The remaining clades (clade A, clade C-I) of vertebrate serpins are extracellular serpins, of which the largest clade is clade A [8]. Clade C serpins topologically belong to a monophyletic clade that separates the last common ancestors of clade B and other clades (A, D-I). Clade C serpins were the first to appear extracellularly and most likely bridge intracellular and extracellular serpins.

Interestingly, some reports indicated that secretory serpins such as PAI-1 [9], SERPINA2 [10], and SERPINH1 [11] are localized in various subcellular compartments. Recently, a study showed that secretory PAI-1 has been found in the Promyelocytic Leukemia Nuclear Bodies (PML bodies) of endothelial cells and might regulate the endothelial cell growth [9]. The unconventional localization of such serpins tantalized us to explore whether the other members of the human serpin superfamily were also inside the cells. If yes, are they localized in the nucleus or other cell organelles?

To reside in the nucleus, a protein requires a nuclear localization signal (NLS), a basic residue-rich motif responsible for the nuclear import of protein [12]. NLSs are basic amino acid-rich (R and K) short peptides that mediate the nuclear import of proteins by binding to their receptors, known as importins (karyopherins). The cargo protein forms a complex with importin α , importin β 1 [13,14]. Importin α serves as an adaptor that links cargos and importin β 1 and recognizes NLSs within the cargos [13,14]. The classical best-characterized NLSs (cNLS) are either monopartite, a single cluster of basic residues, or bipartite, two clusters separated by a stretch of 4-10 residues. Additionally, nonclassical NLSs were also reported earlier [15]. One of the subclasses of nonclassical NLSs is comprised of proline-tyrosine-rich NLS (PY-NLS), which follows the consensus R/K/H(X)₂₋₅PY, and they bind with transportin-1 and 2 (importin Kap β members), mediating the import. These motifs are present in a disordered region of protein possessing an N-terminal stretch of either hydrophobic or basic amino acids and a typical C-terminal R/K/H(X)₂₋₅PY consensus pattern [15].

The nuclear export of proteins is mainly governed by the classical Chromosome region maintenance 1-dependent (CRM1)-dependent nuclear export signals (NES) motifs [16]. Classical CRM1-dependent NES motifs are 10–15 hydrophobic amino acid-long residues (mainly leucine-rich), followed by a specific consensus pattern (Φ 0xx Φ 1x(2,3) Φ 2 x(2,3) Φ 3x Φ 4, where x is any amino acid, and Φ are hydrophobic amino acids), and are categorized into various classes [17,18]. Various studies showed that in addition to the consensus patterns, the NES motifs should be located in the disordered region of the protein for better accessibility to bind with CRM-1, and possess a helical or helix-to-extended confirmation [17].

In this study, we showed for the first time that various secretory serpins from clades A, D, E, H, and I are not obligatory extracellular and were also found in different subcellular compartments, such as the nucleus and centrosome. Notably, most of the members of secretory serpins possess either NLS or NES or both, suggesting that they might have crucial nuclear functions. These findings will help in identifying the functions of uncharacterized serpins and might open avenues for future functional studies of secretory serpins.

Materials and Methods

Collection of Primary Sequences and 3D Structures of Human Serpin Superfamily Members

Primary reference sequences of human serpins belonging to different clades (Serpins clade A-I) were retrieved from Gene hub, NCBI (<https://www.ncbi.nlm.nih.gov/>), and the Ensemble database

(<https://www.ensembl.org/index.html>) (Table S1). Protein structures were obtained from PDB (<https://www.rcsb.org/>) and the UniProt databases (<https://www.uniprot.org/>) [19] (Table S1). The crystal structures of a few serpins were not available. In such cases, the structures were predicted from AlphaFold and used in the study.

Multiple Sequence Alignment

Multiple sequence alignment (MSA) of human serpins was generated by the Clustal Omega package in Unipro U GENE software 52.0 with the default parameters [20–22]. Further, MSAs were visualized in the Unipro U GENE color theme to analyze the conservation of selected motifs.

Subcellular Distribution Data Retrieval of Human Serpins

Two major subcellular resource databases that are based on high-throughput studies of global subcellular distribution profiles of proteins, i.e., the Human Protein Atlas database (HPA) (<https://www.proteinatlas.org/>) (Table S3) [23] and Subcell Barcode (www.subcellbarcode.org) [24], were used to analyze the intracellular localization of human serpins.

NLS and NES Analysis

cNLS mapper, ELM database (<http://elm.eu.org/>), and manual curation methods were used to identify NLSs in human serpins. In the cNLS mapper (<https://nls-mapper.iab.keio.ac.jp/cgi-bin/>), a cut-off score of 0.6 was used to identify NLSs [13]. We manually searched for basic amino acids (R and K) clusters to identify classical NLS and non-classical NLS (PY-NLS). NESs were analyzed using the ELM server (<http://elm.eu.org/>) and the LocNES tool (<http://prodata.swmed.edu/LocNES>). NES having a cut-off score > 0.1 were selected from the LocNES predicted motifs for further analysis. The serpin sequences were also manually analyzed by searching the consensus patterns and structural features of different NES classes described in Method S1-2 [25].

Assessment of the Solvent Accessibility of the NES Motif

The solvent-accessible area of identified NESs was assessed using BIOVIA Discovery Studio 2024 Client Visualizer (<https://www.3ds.com/products/biovia/discovery-studio>) [26]. Serpins were analyzed using the software to calculate the residue solvent accessibility (RSA), sidechain solvent accessibility (SSA), i.e., restricted to side chains, and percent solvent accessibility (PSA). PSA was calculated with a probe radius of 1.4 Å and 240 grid points. The identified motifs showing PSA >25 % were considered solvent-accessible and surface-exposed, while those with PSA <10% were considered buried.

Cell Culture and Immunofluorescence Staining

The human umbilical vein endothelial cells (HUVECs) were isolated from the umbilical cords as described previously [7] as per the guidelines and approval of the Institutional Ethical Committee at the J.L.N. Medical College, Ajmer, India, and the University of Greifswald, Germany. HeLa cells were cultured in DMEM media supplemented with 10% fetal bovine serum and cultured at 5% CO₂ and 37°C. Immunofluorescence studies were done as described earlier [9]. Briefly, cells were fixed for 10 min with 3.7% PFA, permeabilized for 20 min using 0.2% Triton-X100 in PBS/T, and stained with Hoechst 33258 at a 10 µg/ml concentration. Non-specific binding was blocked with 2% fatty acid-free BSA in PBS/T for 40 min, followed by an incubation of the primary antibodies in 2% fatty acid-free BSA in PBS/T for 60 min. Secondary antibody incubation was performed using 2% fatty acid-free BSA in PBS/T for 45 min.

The primary antibodies were used at the dilutions: PAI-1 (C-09) antibody (1:75), PML antibody (1:150). The secondary antibodies were used at a dilution of 1:200. Images were captured using a Zeiss fluorescence microscope with Zeiss Axio Vision 4.8 software for data analysis.

Mitochondrial Isolation and Western Blotting

Hela and HUVEC cells' mitochondria were isolated using the Qproteome Mitochondrial Isolation Kit from Qiagen as per the manufacturer's instructions. In brief, cell pellet of 1×10^7 cells were resuspended in ice-cold lysis buffer and incubated for 10 min at 4 °C. The lysate was centrifuged, and then the cell pellet was resuspended in 1.5ml ice-cold disruption buffer. Cell disruption was completed by passing the cell pellet through a blunt-ended needle several times. The supernatant was collected after centrifugation of the lysate at 1000xg for 10 min at 4 °C. and then centrifuged again at 6000xg for 10 min at 4 °C. The mitochondrial-containing pellet was collected, washed, resuspended, and stored in mitochondrial storage buffer. The mitochondrial samples were prepared using 2X Laemmli buffer, and Western blotting was performed with PAI-1 mouse (C-09) antibodies as described before [7].

Results

Various Secretory Serpins from Different Clades are Not Obligatory Extracellular and Are Found to Be Localized Inside the Cells

Out of 36 human serpins (Table S1), immunofluorescence data of 16 serpins in various cell lines were found in HPA and retrieved (Table 1, Table S3). Interestingly, the secretory serpins from different clades (A1, A10, H1, I1, D1, and E2) were intracellularly localized (Figure 1A). In agreement with the previous reports, the Clade B serpins (B1, B3-B10, and B13) were also found to be intracellular (Figure 2). For further investigation, the subcellular localization information of 20 serpins was retrieved from SubCell BarCode, in which clade A secretory serpins (A4, A6, A7, and A10) and the serpins from other clades (F1 and G1) were found in the cytosol (Table 1). Consistent with HPA data, serpins D1, E2, H1, and I1 were also found intracellularly. Additionally, classical intracellular serpins of Clade B (serpin B1-4, B8, B9, and B11-B13) were localized inside the cells (Table 1). A total of 11 serpins (A2, A3, A5, A8, A9, A11, A12, C1, E3 F2 and I2) were not found in either database. These data showed for the first time that various secretory serpins from different clades are not obligatory extracellular and might have novel intracellular functions.

Secretory PAI-1 was previously reported to be present in the nucleus of endothelial cells [9]. Here, to explore the subcellular distribution of PAI-1 in HeLa cells. Indeed, we identified that PAI-1 is present in the nucleus, especially in the PML bodies (Figure 1B). Interestingly, in the SubCell Barcode database, the PAI-1 protein was also identified in the mitochondrial fraction in the U251 cell line. To verify the localization of PAI-1, we isolated the mitochondria from HUVECs and HeLa cell lines. Interestingly, PAI-1 was observed in the mitochondria of HUVECs but not in HeLa cells. It suggests that mitochondrial localization of PAI-1 is highly regulated and dependent on cell type and specific environment.

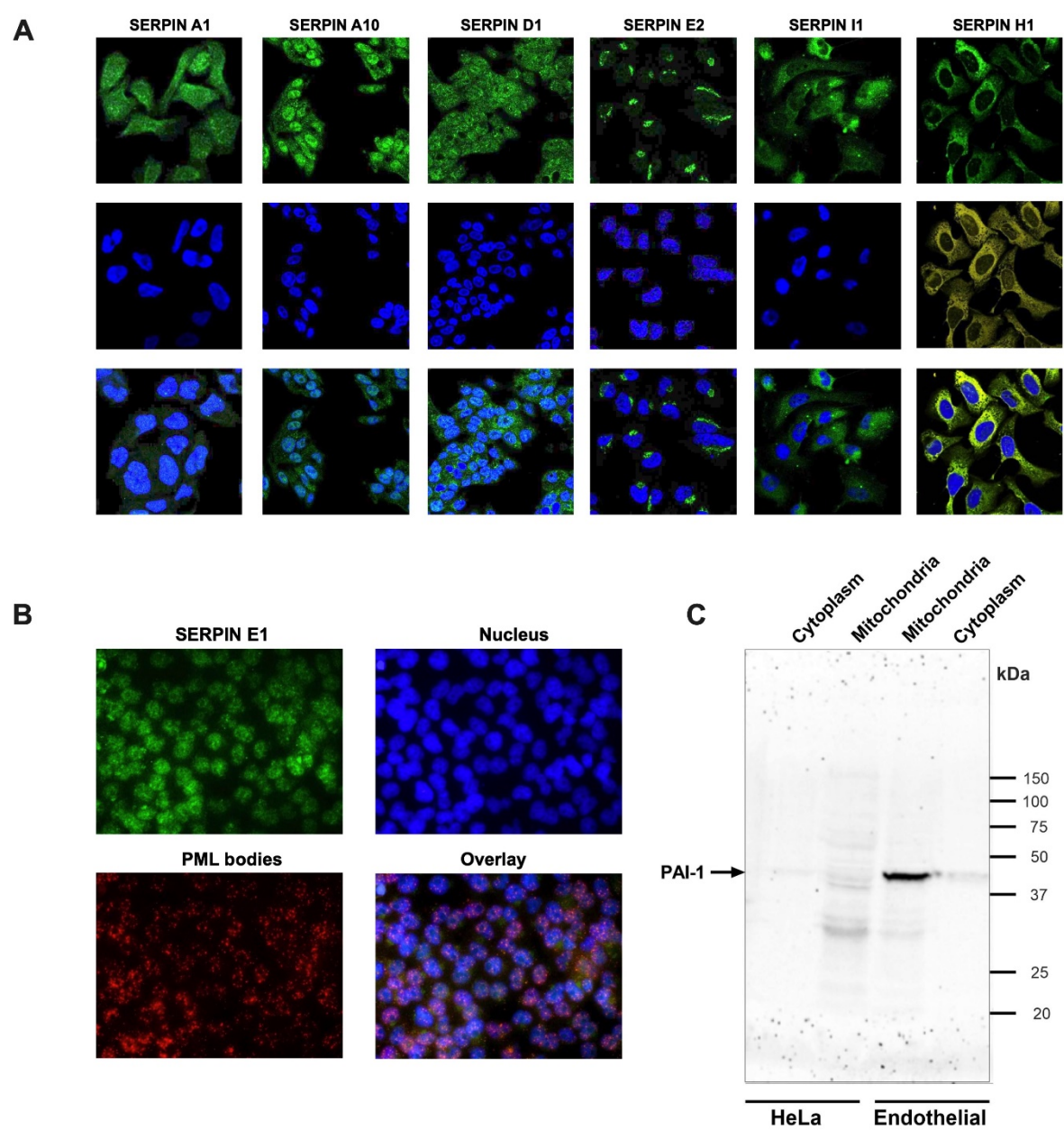


Figure 1. Subcellular localization of various extracellular serpins. A) The immunofluorescence confocal images were retrieved from the HPA subcellular resource. The upper panel (green) shows the staining of the respective serpins. Middle panel: the cell nuclei were stained with DAPI (blue), and the ER was stained with calreticulin antibody, which is shown in yellow. The overlay is shown in the lower panel. B) HeLa cells were stained with specific PAI-1 antibody (left panel, green), nuclei with Hoechst 33258 (middle panel, blue), PML bodies with specific PML antibody, and overlay picture (right panel). PAI-1 was found in the nucleus. C) The mitochondria of HUVECs and HeLa cells were isolated, and then a Western blot was performed using the PAI-1 antibody. A band of PAI-1 is observed in the mitochondria of HUVECs but not in HeLa cells.

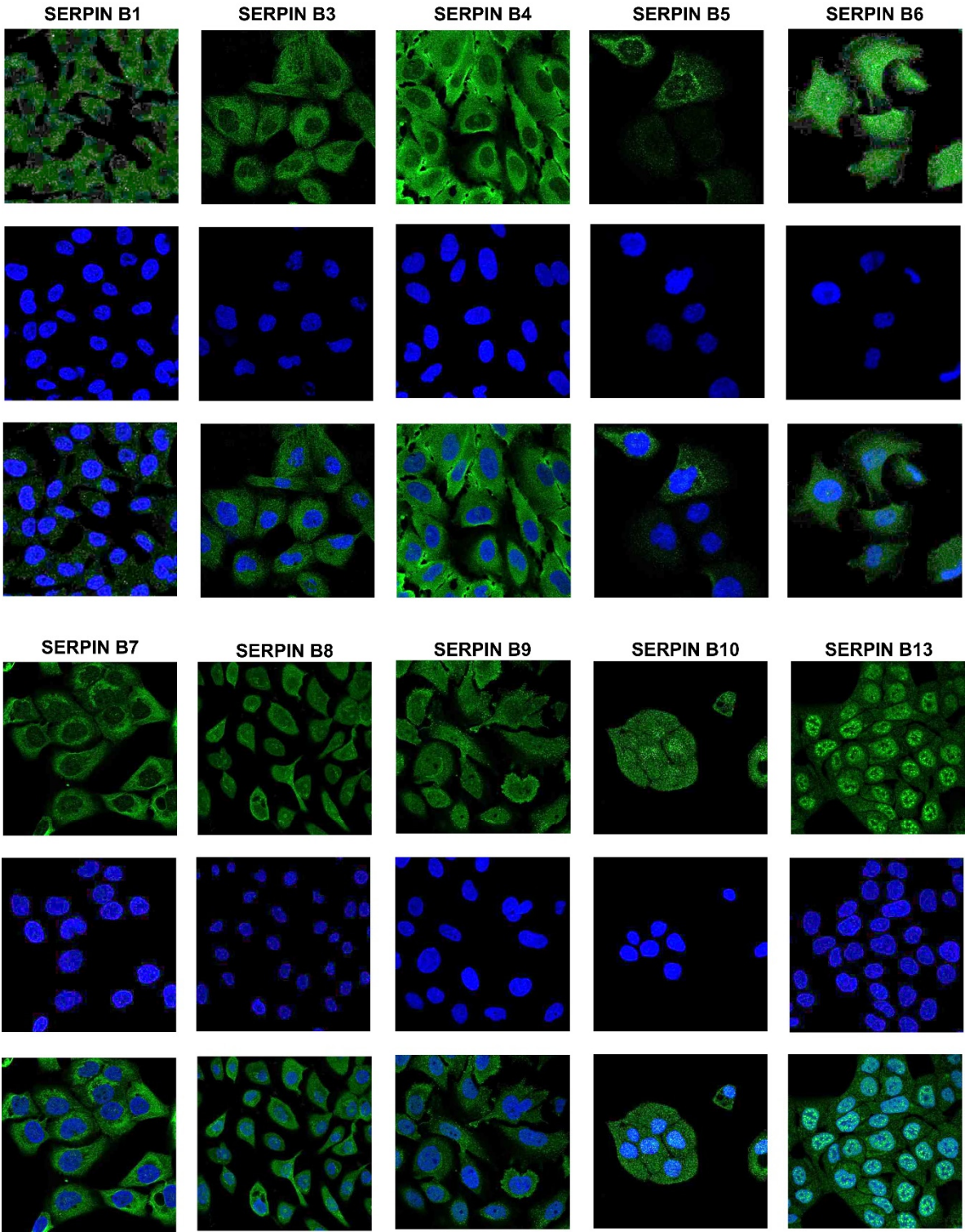


Figure 2. Subcellular localization of Clade B serpins. The immunofluorescence confocal images were retrieved from the HPA subcellular resource. The upper panel (green) shows the staining of the respective serpins. Middle panel: the cell nuclei were stained with DAPI (blue). The overlay is shown on the lower panel.

Various Serpins are Localized in the Nucleus and Have Potential Nuclear Localization Signals

This study found that serpins A10, B2, B8-B11, B13, and H1 are mainly localized in the nucleus, at least as shown by one subcellular resource (Table 1), suggesting that these serpins might have a nuclear localization signal (NLS). We used the ELM database and the cNLS mapper tool to identify NLS in human serpins. Also, we manually screened for basic amino acids (R and K) clusters in the human serpins' primary sequences to identify classical NLSs. We identified a single classical NLS

motif in serpin B2. Two motifs were identified in A10 and B5 (Table 2). Using the ELM server, we couldn't identify any NLS in these identified nuclear serpins (Table 2). These data showed that the identified nuclear serpins A10, B2, and B5 had potential classical NLS motifs. Interestingly, these tools couldn't identify NLS in serpins B8-B11, B13, and H1. To explore this further, we manually analyzed the serpin sequences. We found one basic amino acid-rich NLS motif in serpin B8 and B13 and two NLS motifs in B10 (Table 2), suggesting that these serpins have potential classical NLS.

We also found a specific nonclassical NLS (PY-NLS) in 8 serpins (A2, A3, A5, A10, A12, B1, B3, and E1). Previously, we reported that serpin E1 (PAI-1) didn't have any classical NLS [9]. Notably, in this study, we identified a nonclassical PY-NLS motif in serpins A3 and E1. None of the used methods was able to identify a potential NLS in serpin B9, B11, and H1, suggesting that these serpins may be transported to the nucleus via an indirect mechanism, such as by interacting with NLS-containing proteins. Interestingly, we found that 9 serpins (A2, A3, A5, B1, B3, B4, D1, E1, and I1) had potential NLSs. But, these serpins were not shown in the nucleus by any subcellular databases (Table 2). Notably, the serpins B1, B3, B4, D1, and I1 were localized in the cytosol (Table 1). Additionally, we found that serpin A9 and A12 have potential NLS; however, we couldn't find the subcellular data from the databases. Serpins A5 contains 2 classical NLS motifs, and serpins B1 and I1 have a single classical NLS motif identified using the ELM server. Serpins B1 had 4 NLS (3 classical NLS and 1 nonclassical NLS). Serpin A3 and E1 had one nonclassical NLS motif. Serpin A9 (2 motifs), B4 (1 motif), and D1 (1 motif) had classical NLS. Serpin I1 had one NLS predicted by the ELM database only. Serpin A2 and A5 had 2 classical NLSs and 1 nonclassical NLS. A12 has 1 classical NLS and 2 nonclassical NLSs. B3 has 1 classical NLS and 1 nonclassical NLS. These data suggest that the localization of these serpins is highly regulated and may require specific signaling to import them into the nucleus using identified potential NLSs.

Identification of Potential Nuclear Export Signals in the Serpin Superfamily

Here, we used the ELM database and the LocNES tool to identify NESs in human serpins, followed by manual curation of the predicted NES motifs. The LocNES tool predicted multiple putative NES motifs in 24 human serpins. Notably, the ELM database confirmed the presence of NESs in 18 of these serpins, with the exception of six (B1, E3, I1, I2, A10, and A11), for which no NES motifs were identified. This substantial overlap highlights the robustness of the predictions while also underscoring tool-specific differences. This divergence may be attributed to differences in the underlying prediction algorithms and sensitivity thresholds of each tool. (Table S2).

All identified motifs were subsequently subjected to manual curation, and those that failed to meet the established criteria for known NES classes were excluded from the final analysis. It has been depicted that the NES should follow consensus patterns, possess solvent-accessibility, and have a helical/coiled secondary structure [17]. The secondary structure and surface accessibility of the predicted NES motifs were analyzed within the structural models of human serpins. NES motifs identified in four serpins (B3, B9, B11, and I2) exhibited a solvent-accessible surface area (PSA) of less than 25%, suggesting they are likely buried and not accessible on the protein surface. Additionally, these motifs adopted β -sheet-like conformations, which deviate from the typical helical/coil structure commonly associated with functional NESs. Hence, these motifs were rejected. In the remaining 20 serpins from/assigned to different clades, a total of 27 NES motifs were retained, as they met all established criteria for potential NES (Table 3). The surface accessibility scores and the secondary structure conformations of these selected NESs are shown in Figure S1. No NES motifs were identified in serpins belonging to clades D, F, and G. Interestingly, we found that 10 serpins (A4, A6, A11, B6, B7, B12, C1, E2, E3, and H1) contained NES motifs but lacked identifiable NLS motifs. Another group of 10 serpins (A10, B1, B2, B4, B5, B8, B10, B13, E1, and I1) harbored both NES and NLS motifs, whereas seven other serpins (A2, A3, A5, A9, A12, B3, and D1) possessed only NLS motifs (Table 4). These findings suggest that this subset of serpins may actively shuttle between the nucleus and cytoplasm, potentially engaging in specific functions in the nucleus.

Conservation of Identified NES Motifs in Human SERPIN Superfamily

We investigated whether the identified NES motifs are conserved across members of the human serpin superfamily. Interestingly, multiple sequence alignments (MSA) of human serpin sequences revealed that most NES consensus motifs were conserved and localized within the same regions across the majority of serpins. Notably, each conserved region contained only one type of NES consensus class. Based on this observation, we classified these regions into six distinct groups (Group A to Group F) (Figure 3). In addition, several NES motifs were found in serpins that did not align with any of the defined groups. These were categorized separately as unique groups, referred to as Group U. Among all groups, Group F emerged as the most prominent and conserved region, encompassing 11 of the 27 predicted NES motifs. In comparison, Group U contained six NES motifs (Table 3). Group F is characterized by a typical class 1a NES consensus pattern ($\Phi 0xx\Phi 1xxx\Phi 2xx\Phi 3x\Phi 4$) and is located in the region of 270-370 amino acids.

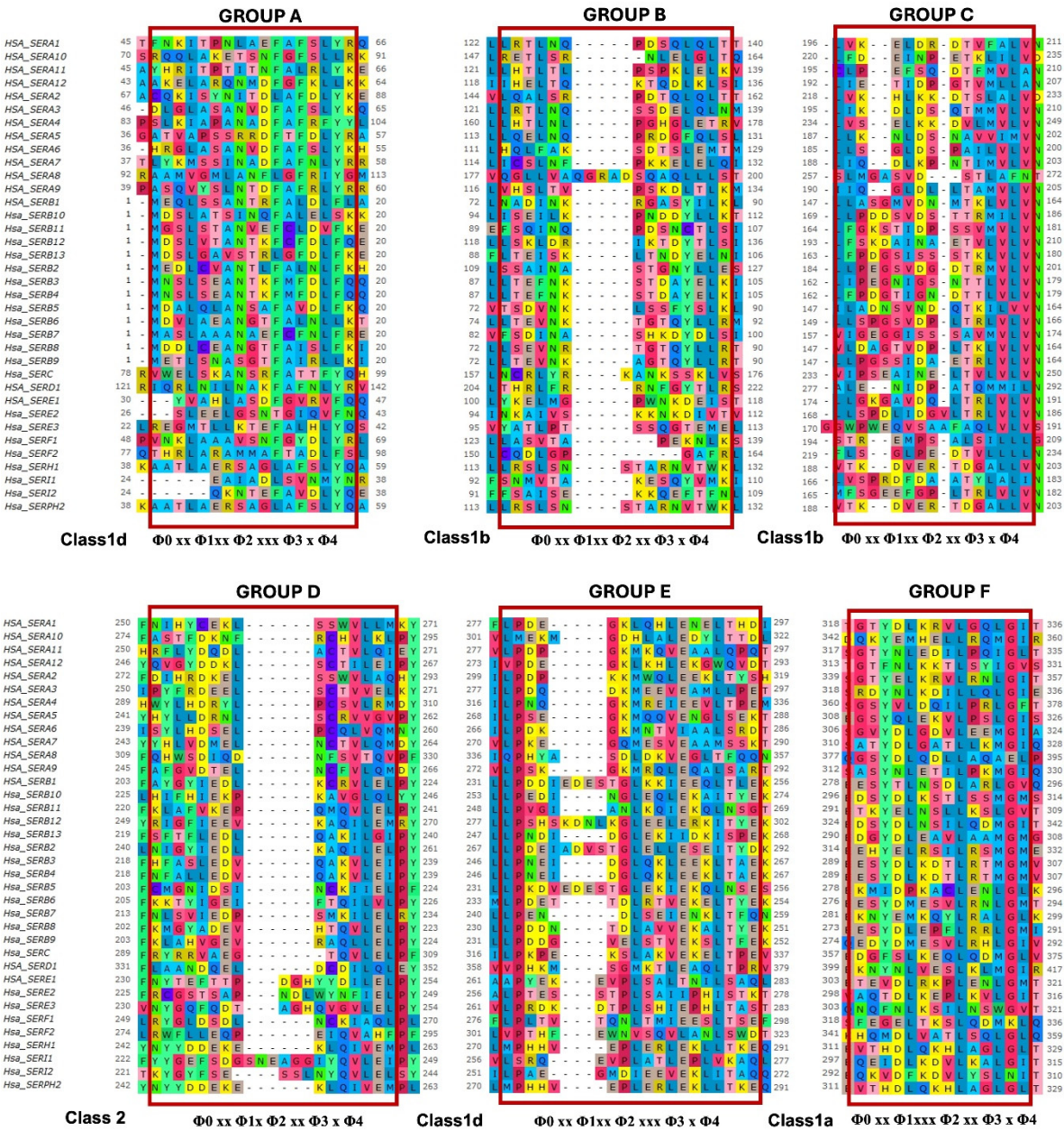


Figure 3. The multiple sequence alignment of NES motifs from clades A-I of human serpins. The multiple sequence alignment of identified NES motifs in members of the serpin superfamily. The sequences of the identified NES motifs in human serpins from clades A-I of vertebrates were aligned. The specific class of

consensus pattern in each NES motif is shown here. The conserved hydrophobic amino acids are shown in boxes. Notably, most of the NES consensus sequences were conserved, and only one type of NES consensus class was present in one region. These regions are divided into 6 groups (group A to group F).

Discussion

It is believed that vertebrate serpins, except Clade B, function exclusively in the extracellular environment [2,27,28]. The bioinformatic analysis of human serpin superfamily members revealed that Clade B members may shuttle between the nucleus and cytoplasm. This study identifies the unique behavior of many extracellular serpins to be present inside the cell, e.g., the mitochondria, nucleus, and possesses either NLS or NES or both. The subcellular localization of secretory serpins in the cells gives a strong hint about their intracellular functions and alternative protein trafficking, which might be secretory signal-independent and regulated by novel regulatory pathways.

We explored two major subcellular resource databases based on high-throughput studies of global subcellular distribution profiles of proteins, i.e., the HPA and Subcell Barcode [23,24]. In the HPA database, we could retrieve the immunofluorescence data of 16 serpins. Surprisingly, we found that various secretory serpins from clades A, D, E, H, and I are not obligatory extracellular and are present inside the various subcellular compartments of the cells. Notably, the identified secretory serpins were not only found in the cytoplasm and nucleus but also in other subcompartments such as mitochondria, cytoskeleton, and ER. Interestingly, PAI-1 was present in the mitochondria of endothelial cells but absent in HeLa cells, suggesting its cell-specific intracellular localization of PAI-1 that should be explored in future.

Moreover, the subcellular localization data of Serpin A1, A2, and I1 were unavailable in either subcellular resource. Some previous studies showed that they might be present in the ER [10,29], but we couldn't detect the classical ER motifs in these serpins. Serpin A2 was co-localized with CANX and PDIA3, two chaperones involved in ER quality control processing [30,31]. Interestingly, in COS cells, serpin I1 (neuroserpin) was reported in the ER or Golgi bodies [10]. These data suggest that these serpins might have functions in the ER and the Golgi.

Together, these data suggest the functions of these serpins inside the cell, and they might regulate cellular functions such as cell adhesion [32], apoptosis [33], and cell growth [9]. It was intriguing that the intracellular localization of secretory serpins is not in line with the evolution-based origin of secretory serpins, suggesting that the secretory serpins acquired additional extracellular functions during evolution, in addition to the intracellular functions, due to an increase in body complexity and biological functions. The novel regulatory pathway may have evolved to coordinate its sub-cellular distribution in accordance with specific cell types and a cell state-dependent manner.

In this study, we observed that most of the members of clade B and a few secretory serpins are mainly localized in the nucleus, at least as shown by one subcellular database. Most nuclear serpins contain basic residues (R, K) rich motifs with specific conserved patterns of NLS motifs. NLS analysis revealed that, except for serpin H1, we could identify NLS in all the identified nuclear serpins (Table 2), which indicates that they have an inherent property to localize in the nucleus. Consistent with previous studies, our findings also show that most intracellular serpins, particularly those from clade B, contain identifiable NLS motifs and are predominantly localized in the nucleus [34–36]. Surprisingly, we could not identify any NLS in serpin B9 (PI-9). Still, it is actively imported into the nucleus (Table 1) [37], suggesting that PI-9 may interact with an NLS-containing protein to enter the nucleus. It is unlikely that PI-9 passively diffuses in the nucleus due to its molecular weight (~40 kDa) [14,37,38].

Notably, some of the members of secretory serpins contain the potential NLS sequences. It is surprising that these secretory serpins were not only found inside the cells but also tend to shuttle between the nucleus and the cytoplasm, suggesting their functions in the nucleus that are yet to be explored. Previous reports showed that the nuclear localization of serpin A3 depended on the N-glycosylation pattern [39,40]. In this study, we found a potential PY-NLS motif in serpin A3, suggesting that N-glycosylation might help in translocating it into the nucleus.

We identified 27 NES motifs in 20 human serpins. Remarkably, 10 serpins possess only NES motifs, and the other 10 serpins contain both NLS and NES motifs. Serpin A4 and A6 have only NES and are found in the cytosol and the ER, respectively (Table 1). Also, it is interesting to note that serpins B6, B7, and B12 are found in the centrosome, ER, and mitochondria, respectively, and contain only NESs. Other secretory serpins, serpin A1, I1, and E1, have an N-terminal signal peptide, and we found that they contain both NLS and NES motifs, indicating that they might have functions in the nucleus. Furthermore, we could identify 7 serpins that possess only NLS and were not found in the nucleus. In subcellular resources, few serpins were cytosolic (Table 1) but had at least one potential NLS (Table 2). It is also observed in other proteins, such as LIMK1 and LIMK2, which possess functional NLS but are mainly localized in the cytoplasm [41]. The possible explanation is that these proteins might shuttle between the nucleus and cytoplasm, and active NES motifs are responsible for their cytoplasmic presence. Also, NLS might be exposed in specific conditions, and the protein may be localized in the nucleus.

Notably, we identified 6 regions (Group A to F) in serpins sequences where most identified NESs were present and possess a region-specific consensus class of NES. It was very interesting to observe that amongst these identified groups, group F contains the highest number of NES motifs from different serpins. Therefore, Group F is the largest group found in the region of 270-370 amino acids and follows the “1a” consensus pattern (Table 3), suggesting that this conservation might have an evolutionary significance.

In conclusion, this study might open avenues for future functional studies of secretory serpins in various physiological conditions and diseases. Some serpins (Table 5) have functions that do not require protease inhibition and have paradoxical physiological and pathophysiological functions. These secretory serpins identified inside the cells may be used as a reference in identifying the intracellular functions to decipher the precise role in various human diseases.

Table 1. Subcellular localization of human serpins. The subcellular distributions of human serpins were analyzed using the subcellular resource of the Human Protein Atlas (HPA) and Subcell Barcode resources. PM: plasma membrane; ER: endoplasmic reticulum.

S.No.	Clade	Protein code	HPA	Cell line	Subcell Barcode	Cell line	Literature
1.	A	SERPINA1	Vesicles	HepG2	N/A	N/A	ER [10]
2.		SERPINA2	N/A	N/A	N/A	N/A	ER [10]
3.		SERPINA3	N/A	N/A	N/A	N/A	Nucleus [40], [39]
4.		SERPINA4	N/A	N/A	Cytoplasm	MCF7	N/A
5.		SERPINA6	N/A	N/A	Cytoplasm	MCF7	N/A
6.		SERPINA7	N/A	N/A	Cytoplasm	MCF7	N/A
7.		SERPINA10	Cytosol, Nucleus	HepG2	Cytoplasm	H322	N/A
8.	B	SERPINB1	Cytoplasm	SK-MEL-30	Cytoplasm, cytoskeleton	H322	N/A
9.		SERPINB2	N/A	N/A	Cytoplasm	A431, H322	Nucleus [34]
10.		SERPINB3	Cytoplasm, PM	A431	Cytoplasm	H322	N/A
11.		SERPINB4	Cytoplasm, PM	U2OS	Cytoplasm	H322, HCC827	N/A
12.		SERPINB5	Vesicles	A431	Cytoplasm	A431, H322, MCF7, HCC827	Nucleus [35,36,42]
13.		SERPINB6	Centrosome	U251-MG	NA	A431	Centrosome [43]
14.		SERPINB7	Mitochondria, ER	HaCaT	NA	H322	N/A
15.		SERPINB8	Golgi bodies, Nucleus	HBEC3KT	Cytoplasm, cytoskeleton	A431, HCC827	N/A
16.		SERPINB9	Cytoplasm, Nucleus	SiHa	Cytoplasm, cytoskeleton	HCC827	Nucleus, cytoplasm [37]
17.		SERPINB10	Cytoplasm, Nucleus	HaCaT	N/A	N/A	N/A

18.		SERPINB11	N/A	N/A	Nuclear	HCC827	N/A
19.		SERPINB12	N/A	N/A	Mitochondria	HCC827	N/A
20.		SERPINB13	Cytoplasm, Nuclear speckles	RT-4	Cytoplasm	H322	N/A
21.	D	SERPIND1	Vesicles	HepG2	Cytoplasm	U251	N/A
22.	E	SERPINE1	N/A	N/A	Mitochondria, ER	U251	Nucleus [9]
23.	E	SERPINE2	Golgi bodies	SK-MEL-30	PM	U251	N/A
24.	F	SERPINF1	N/A	N/A	Cytoplasm cytoskeleton	H322	N/A
25.	G	SERPING1	N/A	N/A	Cytoplasm	H322, MCF7	N/A
26.	H	SERPINH1	ER	U2OS	Nucleosol, ER	U251, A431	ER [11,44]
27.	I	SERPINI1	Cytoplasm, vesicles	U251-MG	N/A	N/A	ER, Golgi bodies, vesicles [29]

Table 2. Prediction of NLS motifs in human serpins based on primary sequence analysis. The cNLS Mapper tool, ELM database, and manual curation method were used to identify putative NLS motifs in all human serpins.

S. No.	Name	Position	cNLS Mapper		ELM Database	Manual curation	
			Sequence	Score		Classical NLS	Non classical NLS (PY-NLS)
1	SERPINA2	11-16	RNLGITKIFSNEADLSGVSQEAPLKLSKA	5.3	N/A	EKRTGRKVVDLVKHLKKD	HRLGPY
		350-378					
		203-220					
2	SERPINA3	247-253	N/A		N/A		HHLTIPY

3	SERPINA5	254-260 294-300 292-299	N/A		FKKRQLE KMFKKRQL		RVVGVPY
4	SERPINA9	171-189 280-301	N/A		N/A	AQARINSHVKKKTQGKVV RQLEQALSARTLRKWSHSLQKR	
5	SERPINA10	179-206 238-267 286-292	FNLSKRYFDTECVPMNFRNASQAKRLMN FKGKWLTPFDPVFTEVDTFHLDKYKTIKVP	5.4 6.4	N/A		HVLKLPY
6	SERPINA12	39-66 339-345 385-390	WKQRMAAKELARQNMDFGFKLLKKLAFN	5.35	N/A	N/A	LTKIAPY KIDKPY
7	SERPINB1	168-197 189-205 185-200 215-221	KGNWKDKFMKEATTNAPFRLNKKDRKTVKM	5.1	KKDRKTVKMMYQKKKFA	FRLNKKDRKTVKMMYQ	RVLELPY
8	SERPINB2	143-172 165-181	RLCQKYYSSEPQAVDFLECAEEARKKINSW	6.3	N/A	ARKKINSWVKQTQKGKI	
9	SERPINB3	143-160	N/A		N/A	SRKKINSWVESQTNEKIK	

		229-236					AKVLEIPY
10	SERPINB4	142-150	N/A		N/A	ESRKKINSW	
11	SERPINB5	83-114 87-114	FYSLKLIKRLYVDKSLNLSTEFISSTKRPYAK KLIKRLYVDKSLNLSTEFISSTKRPYAK	5.1 6.1	N/A	N/A	
12	SERPINB8	176-191	N/A	N/A	N/A	RKYTRGMLFKTNEEKK	
13	SERPINB10	73-83 222-233	N/A	N/A	N/A	EKKRKMEFNLS MKKKLHIFHIEK	
14	SERPINB13	143-155	N/A		N/A	ESRKKINSWVESK	
15	SERPIND1	203-219	N/A		N/A	RKLTHRLFRRNFGYTLR	
16	SERPINE1	54-61	N/A		N/A	N/A	RNVVFSPY
17	SERPINI1	283-289	N/A		VKKQKVE	N/A	

Table 3. Summary of the potential NESs identified in the various human serpins.

Clade	Position	Sequence	Serpin	Group	Class	Consensus class
A	116-130	DVHRGEQHL ^U HTL ^U NL ^U	A4	U	1a	$\Phi 0 \times x \Phi 1xxx \Phi 2 \times x \Phi 3 \times \Phi 4$
	118-133	SDTSLEMTMG ^U NAL ^U FL ^U	A6	U	1c	$\Phi 0 \times x \Phi 1xxx \Phi 2 \times x x \Phi 3 \times \Phi 4$
	307-321	SGVYD ^U LGDVLEEM ^U GI ^U	A6	F	1a	$\Phi 0 \times x \Phi 1xxx \Phi 2 \times x \Phi 3 \times \Phi 4$
	300-312	VVLMEKMGDHL ^U AL ^U	A10	U	1d	$\Phi 0 \times x \Phi 1xx \Phi 2 \times x x \Phi 3 \times \Phi 4$
	92-106	QANTSALILEGL ^U GF ^U N ^U	A11	D	2	$\Phi 0 \times x \Phi 1x \Phi 2 \times x \Phi 3 \times \Phi 4$

B	238-252	DESTGLKKIEEQTL	B1	E	1d	$\Phi 0 \times \Phi 1 \times \Phi 2 \times \Phi 3 \times \Phi 4$
	279-293	EESYTLNSDLARLCV	B1			
	259-273	TKPENLDFIEVNVSL	B1	F	1a	$\Phi 0 \times \Phi 1 \times \Phi 2 \times \Phi 3 \times \Phi 4$
	268-282	LPDELADVSTGLELL	B2	E	1d	$\Phi 0 \times \Phi 1 \times \Phi 2 \times \Phi 3 \times \Phi 4$
	292-306	ESYDLKDTLRTMGMV	B4	F	1a	$\Phi 0 \times \Phi 1 \times \Phi 2 \times \Phi 3 \times \Phi 4$
	74-88	TSDVNKLSSFYSKL	B5	B	1b	$\Phi 0 \times \Phi 1 \times \Phi 2 \times \Phi 3 \times \Phi 4$
	149-163	LLSPGSVDPLTRLVL	B6	C	1b	$\Phi 0 \times \Phi 1 \times \Phi 2 \times \Phi 3 \times \Phi 4$
	241-255	LPENDLSEIENKLTIF	B7	E	1d	$\Phi 0 \times \Phi 1 \times \Phi 2 \times \Phi 3 \times \Phi 4$
	283-297	KNYEMKQYLRALGLK	B7	F	1a	$\Phi 0 \times \Phi 1 \times \Phi 2 \times \Phi 3 \times \Phi 4$
	274-288	EESYDLEPFLRRLGM	B8			
	297-311	ENSYDLKSTLSSMGMI	B10			
	305-319	EDSYDLNSILQDMGI	B12			
	218-232	QMMFMKKKLHIEHIE	B10	U	1a	$\Phi 0 \times \Phi 1 \times \Phi 2 \times \Phi 3 \times \Phi 4$
	221-235	SFTFLEDLQAKILGI	B13	D	2	$\Phi 0 \times \Phi 1 \times \Phi 2 \times \Phi 3 \times \Phi 4$
	250-262	NDIDGLEKIIDKI	B13	U	1aR	$\Phi 0 \times \Phi 1 \times \Phi 2 \times \Phi 3 \times \Phi 4$
C	358-372	EDGFSLKEQLQDMGL	C1	F	1a	$\Phi 0 \times \Phi 1 \times \Phi 2 \times \Phi 3 \times \Phi 4$
E	306-318	ETEVDLRKPLENLGM	E1			

	150-164	LSPDLIDGVLTRIVL	E2	U	1a	Φ0 xx Φ1xxx Φ2 xx Φ3 x Φ4
	175-189	DGVLTRIVLVNAVYF	E2	C	1b	Φ0 xx Φ1xx Φ2 xx Φ3 x Φ4
	318-332	VTDLFDPLKANLKGI	E3	U	1aR	Φ0 x Φ1xx Φ2 xxx Φ3 xx Φ4
H	312-326	EVTHDLQKHLAAGLGL	H1	F	1a	Φ0 xx Φ1xxx Φ2 xx Φ3 x Φ4
I	298-312	EQEIDLKDVLLKALGI	I1	F	1a	Φ0 xx Φ1xxx Φ2 xx Φ3 x Φ4

Table 4. Summary of identified NLS and NES in human serpins.

S.no.	Clade	Protein code	NLS	NES	S.no.	Clade	Protein code	NLS	NES
1.	A	SERPINA2	NLS	N/A	15.	B.	SERPINB6	N/A	NES
2.		SERPINA3	NLS	N/A	16.		SERPINB7	N/A	NES
3.		SERPINA4	N/A	NES	17.		SERPINB8	NLS	NES
4.		SERPINA5	NLS	N/A	18.		SERPINB10	NLS	NES
5.		SERPINA6	N/A	NES	19.		SERPINB12	N/A	NES
6.		SERPINA9	NLS	N/A	20.		SERPINB13	NLS	NES
7.		SERPINA10	NLS	NES	21.	C.	SERPINC1	N/A	NES
8.		SERPINA11	N/A	NES	22.	D.	SERPIND1	NLS	N/A
9.		SERPINA12	NLS	N/A	23.	E.	SERPINE1	NLS	NES
10.	B	SERPINB1	NLS	NES	24.		SERPINE2	N/A	NES
11.		SERPINB2	NLS	NES	25.		SERPINE3	N/A	NES
12.		SERPINB3	NLS	N/A	26.	H.	SERPINH1	N/A	NES

13.		SERPINB4	NLS	NES	27.	I.	SERPINI1	NLS	NES
14.		SERPINB5	NLS	NES					

Table 5. List of the secretory serpins that are of interest and recommended for future studies.

S.No.	Clade	Protein code	Localization signal		Functions (protease inhibition-independent) and associated diseases	References
			NLS	NES		
1.	A	SERPINA3	NLS	N/A	Inflammation, apoptosis, cancer diagnosis, Alzheimer's and emphysema	[39,40]
2.		SERPINA4	N/A	NES	Inflammation, Diabetic retinopathy, novel prognostic indicator and therapeutic target for Colorectal cancer.	[45,46]
3.		SERPINA5	NLS	N/A	Coagulation, sperm development, tumor cell invasion and metastasis	[28]
4.		SERPINA6	N/A	NES	Hormone transport	[28]
5.		SERPINA9	NLS	N/A	B cell development	[28]
6.		SERPINA10	NLS	NES	Venous thromboembolic disease	[47,48]
7.		SERPINA11	N/A	NES	Hepatocellular carcinoma	[47,49]
8.		SERPINA12	NLS	N/A	Anti-insulin resistance and Obesity	[28]
9.	E	SERPINE1	NLS	NES	Cell Growth , aging, cancer prognosis	[7,9,50]
10.		SERPINE2	N/A	NES	Neurotrophic factors, emphysema, thoracic aortic aneurysms and atherosclerosis.	[28,46,47,51]
11.	H	SERPINH1	N/A	NES	Rheumatoid arthritis, cancer and aortic stenosis	[11,44]

12.	I	SERPINI1	NLS	NES	Neurotrophic factors	[28]
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Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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