Novel biomarkers containing citrullinated peptides for diagnosis of systemic lupus erythematosus using protein microarrays

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Abstract

Objective
Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterised by autoantibody production. This study aims to identify biomarkers involving citrullinated peptides that can be used for SLE diagnosis.

Methods
After a negative selection step with serum from healthy controls (HCs), a phage library of 12 peptides was used for three rounds of screening with sera from 30 SLE patients. After four rounds of biopanning, 21 positive peptides were sequenced. We produced 37-feature arrays containing 16 recombinant citrullinated peptides. The microarrays were tested with an independent validation set of serum samples from 50 HCs, 60 SLE patients, and 60 rheumatoid arthritis (RA) patients.

Results
Microarray analysis showed that the positive rates of 13S1212Cit3-IgM (60.0%), 13S1210-IgG (43.33%), and 13S1212Cit3-IgG (41.67%) were increased in SLE patients compared with HCs and RA patients. The area under the receiver operating characteristic curve (AUC) was 0.770, 0.687 and 0.698, respectively. The combination of 13S1212Cit3-IgM and 13S1210-IgG (termed COPSLE, for combination of peptides for SLE) was more efficient for SLE diagnosis, with a larger AUC (0.830) and a positive rate of 73.33%. COPSLE could be used to identify 80.0% of SLE patients who were negative for anti-Smith (Sm), anti-double-stranded DNA (ds-DNA), and anticardiolipin (ACA).

The Spearman rank correlation indicated that COPSLE increased with albumin, serum level of C3 and platelet distribution width, but had negative correlations with decreased C3 and discoid lupus.

Conclusion
A citrullinated/non-citrullinated peptide panel is a valuable diagnostic marker of SLE, even for patients who are negative for anti-Sm, anti-ds-DNA and ACA.

Key words
systemic lupus erythematosus, rheumatoid arthritis, biological markers, citrullinated peptides, protein microarrays
SLE diagnosed by citrullinated peptides / Y. Wang et al.

Introduction
Systemic lupus erythematosus (SLE) is a prototype of chronic autoimmune connective tissue disease with an insidious onset that can affect almost every system and organ in the human body (1). So far, >180 autoantibody specificities have been found in the blood of SLE patients (2). Circulating autoantibodies can be detected several years prior to the clinical onset of SLE, and in some patients, the number of distinct autoantibodies increased over time (3, 4). It is conceivable that some of the autoantibodies play pathogenic roles and are associated with the wide spectrum of clinical manifestations in SLE (5, 6). The current revised American College of Rheumatology (ACR) classification criteria for SLE (7, 8) contain four autoantibodies: anti-nuclear antibody (ANA), anti-Smith (Sm) antibody, anti-double-stranded (ds)DNA antibody, and anticardiolipin antibody (ACA). ANA is positive in >90% of SLE patients. However, ANA-positive serum samples can be found in about 20% of the general population (9). Thus, ANA as a serological marker for diagnosis of SLE has been sometimes diminished as a result of its poor specificity (10). Anti-dsDNA antibody is not particularly sensitive because it may be present transiently, and as a result occurs in only 50–60% of lupus patients at some point during the course of their disease (9). Anti-Sm antibodies are found in 5–30% of SLE patients (9). ACA is observed in 16–60% of SLE patients (9). Although anti-dsDNA, anti-Sm and anti-ACA have been used as a universally diagnostic criterion for SLE, as well as for monitoring SLE disease activity including renal and central nervous involvement, they are limited by their poor sensitivity in the identification of SLE disease activity and lupus flares (11-14). Therefore, for the diagnosis of SLE, it is necessary to include other markers with higher specificity and sensitivity. In this study, we used a random phage display library of 12-mer peptides to screen sera from healthy controls (HCs) and SLE patients and obtained peptides. Similar to anti-cyclic citrullinated peptide (CCP) antibody for the diagnosis of rheumatoid arthritis (RA), we synthesised citrullinated/non-citrullinated peptides and compared their reactivity in serum samples for HCs and patients with SLE or RA using protein microarray methods. Protein arrays as high-throughput antibody screening platforms have the potential to distinguish antibody specificities against a wide spectrum of antigens, and are therefore, valuable for the evaluation of correlations between antibodies and clinical manifestations (15-17). We found that the COPSLE (combination of peptides for SLE) is a potential diagnostic marker of SLE; even for patients whose disease is anti-Sm, ds-DNA, and ACA negative. The COPRA (combination of peptides for RA) may be a marker for RA.

Materials and methods

Ethical statement
This study was a retrospective study that was approved by the Ethics Committee of Harbin Medical University (HMU) (approval no.: HMU/IRB20170002) and was carried out in accordance with the Declaration of Helsinki.

Reagents and study population
The phage display peptide library and host strain Escherichia coli ER 2738 were obtained from the New England Biolabs (Ipswich, MA, USA). The library, based on a combinatorial library of random peptide 12-mers fused to a minor coat protein (p III), had 2.7×10⁹ electroporated sequences. Phage titre was 1.5×10¹³ pfu/ml.

Between June 1, 2013 and December 31, 2014, we enrolled 90 Chinese Han ethnic outpatients or inpatients who were classified as having SLE according to the ACR 1982 classification criteria (updated in 1997) from the Second Affiliated Hospital of HMU. Eighty age-matched and sex-matched healthy Chinese Han people attending routine physical examinations and 60 Chinese Han inpatients with RA (American Rheumatism Association criteria) were also recruited. Student’s t-test showed no significant difference in the mean age or sex ratio among SLE, RA and HC participants (Supplementary Table S1). Clinical examination indicators (CEIs) were retrieved from the hospital electronic medical records system.
and examined with reference to the revised ACR classification criteria (8). CEIs included cumulative SLE-related clinical manifestations, hematological disorders, and immunological disorders. Information pertaining to human samples was recorded such that participants could not be identified directly or through linked identifiers. After obtaining informed consent, blood samples were collected from all participants.

**Preparation of sera**

Blood samples were collected in venous blood collection tubes with clot activator and gel for serum separation and centrifuged at 4,000rpm for 10 min. All sera were filtered with microcell filters (<0.22 μm) to eliminate red blood cell fragments and bacteria, and frozen at -80°C immediately. Samples were centrifuged and aliquoted within 48 h.

**Screening of SLE-specific peptides**

Serum pools were obtained from 30 HCs and 30 SLE patients. Microtitre wells were coated overnight at 4°C with 150 μl sera from healthy controls diluted with NaHCO3. Plates were blocked with 3% non-fat milk for 2 h at 37°C, and subsequently washed six times with phosphate-buffered saline with Tween 20 (0.05% Tween 20). A 100-μl diluted random 12-peptide phage display library with a titre of 2.0×10^11 pfu/ml was added to the coated plates. After incubation for 1 h at room temperature, unbound phages were collected and 100 μl was added per well to SLE serum-coated plates. Following incubation for 1 h at room temperature, bound phages were eluted with 100 μl 0.2 mol/l glycine–HCl (pH 2.2) and neutralised with 1 mol/l Tris-HCl (pH 9.1). Eluted phages were amplified in a host strain and purified by precipitation for about 4 h using one-sixth volume of polyethylene glycol/NaCl. Another two rounds of SLE-serum affinity selection were carried out using the same process. Percentage enrichment was calculated using the formula: percentage enrichment of phage clones (%) = (eluted phages/added phages) ×100%.

**Phage DNA sequencing**

Positive phage clones were precipitated with polyethylene glycol/NaCl. Phage DNA was extracted for sequencing with a QIAprep Spin M13 kit and sequenced with an ABI PRISM 377 sequencer using the -96 gIII sequencing primer (5'-CCC TGA TAG TTA GCG TAACG-3') from the Phage Display Peptide Library kit.

**Production and processing of microarrays**

Microarrays were assembled from 21 peptides previously selected from the phage-display library and 16 recombinant citrullinated peptides. Lysates were dried, blocked with 3% (w/vol) bovine serum albumin in phosphate-buffered saline for 2 h, washed four times in Tris-buffered saline (TBS; 0.5% Tween 20) for 15 min, and incubated with 100 μl 1:10 diluted sera from 60 SLE patients, 60 RA patients and 50 HCs overnight at 4°C. After five washes in TBS (0.5% Tween 20), microarrays were incubated for 1 h at 37°C with a 1:1000 dilution of two detection antibodies mixed together: goat anti-human IgG Cy3-conjugated, and goat anti-human IgM Cy5-conjugate (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Subsequently, they were washed five times in TBS (0.5% Tween 20), rinsed with distilled water and dried by centrifugation. Reference serum was included in each series of experiments. Arrays were scanned using GenePix4000B and the results recorded as a gpr file. Data were extracted using Gene Pix software.

**Microarray data processing**

Cy5 and Cy3 signals were determined for each spot by subtracting background median values from foreground mean values and adding 10. For results below 10, a minimal value of 10 was assigned. The geometric average of duplicates was used as the final signal intensity for a given protein. Spots that did not pass quality criteria (morphologically heterogeneous spots and spots that differed by >50% between replicates) were excluded from analysis. For microarray normalisation, individual signal intensities were further divided by total signals of the microarray. The mean value of a given peptide for all control serum samples was calculated; peptides in an individual serum with >2-fold signal intensity increase were considered positive. The number of positives per peptide in patient and control sera was counted (18).

**Statistical analysis**

Chi-square tests (Fisher’s exact tests) were used to compare positive rates between groups. SLE-specific biomarkers were identified using the following criteria: positive rate ≥30% in patient sera and p<0.05. We conducted multivariate logistic regression to increase diagnostic accuracy. Odds ratio (OR) >1 represented a risk factor, while <1 was interpreted as a protective factor (19). MedCalc v. 15.8 was used to perform pairwise comparisons of receiver operating characteristic (ROC) curves (20). An area under the ROC curve (AUC) of 0.5 indicated chance performance; AUCs of 0.5–0.6 indicated poor predictive ability; AUCs of 0.6–0.7 indicated sufficient predictive ability; AUCs of 0.7–0.8 indicated good predictive ability; AUCs of 0.8–0.9 indicated very good predictive ability; and AUCs of 1.0 indicated excellent predictive ability (21). Spearman rank correlation was used for comparison of peptide data with clinical parameters. Data are presented as mean ± standard deviation. All statistical calculations were performed with SAS v. 9.3 (Serial 989155, SAS Institute Inc., Shanghai, China). Differences were considered as statistically significant when p<0.05.

**Results**

Screening of SLE-specific peptides

After negative selection with HC serum, a phage library of random peptide 12-mers was screened with SLE patient sera (Table 1), as three rounds of bio-panning followed by immunoscreening of enriched libraries. The percentage enrichment of phage increased from 3.3×10^{-4} to 10^{-1}%, a nearly 300-fold enrichment. Data indicated successful affinity selection of phage that specifically bound sera from SLE patients.
Analysis of phage DNA sequences and generation of microarrays
After four rounds of biopanning, 21 positive peptides were obtained and sequenced. We fabricated 37-feature arrays containing 16 recombinant citrullinated peptides derived from the 21 positive peptides (Table II, patent pending). A panel of 16 citrullinated peptides and 21 phage peptides previously selected from a phage display 12-peptide library was assembled and used for microarrays production.

Validation of microarrays as SLE diagnostic biomarkers
To define antibodies with potential diagnostic significance, a 37-feature microarray was tested with sera from 60 patients with SLE, 50 age-matched and gender-matched healthy donors and 60 patients with RA (Suppl. Table S2). After excluding low-quality spots and correcting for variations in phage quantity and differences across print runs, an individual cut-off discriminating between sera-positive and sera-negative samples was calculated for each antigen. The 74 different isotypes of antibodies, including IgG and IgM, reacted with the sera to give a positive rating. Specific antigen candidates were identified using a positive rate ≥30% in sera and p<0.05 (Suppl. Table S3). Chi-square tests indicated that <0.0001, 0.0001 and 0.0001, RA (p=0.0005, 0.0342 and 0.0102). 13S1212Cit3-IgM efficiently discriminated sera from SLE patients than 13S1212-IgM, 13S1212Cit1-IgM and 13S1212Cit2-IgM (p<0.0001, 0.0001 and 0.0001) (Fig. 1A). 13S1213Cit3-IgM efficiently discriminated sera from SLE patients than 13S1212-IgM, 13S1212Cit1-IgM and 13S1212Cit2-IgM (p<0.0001, 0.0001 and 0.0001) (Fig. 1B). 13S1210-IgG efficiently discriminated sera from SLE patients than 13S1210Cit-IgG, (p=0.0001) (Fig. 1C).

Combination of peptides increased SLE diagnostic accuracy
We conducted multivariable logistic regression analysis using three peptides (13S1212Cit3-IgM, 13S1210-IgG and 13S1212Cit3-IgG) in SLE. Results indicated that COPSLE, i.e. combination of 13S1212Cit3-IgM, with OR 19.132 [95% confidence interval (CI) 5.125–71.428, p=0.0001], and 13S1210-IgG, with OR 8.678 (95% CI 2.167–34.757, p=0.0023), was a potential biomarker for SLE diagnosis. Adding 13S1212Cit3-IgG to the combination did not improve the diagnosis ratio of SLE, suggesting an overlapping component of 13S1212Cit3-IgG with 13S1212Cit3-IgM and 13S1210-IgG for diagnostic value. ROC curve analyses of 13S1212Cit3-IgM, 13S1210-IgG and 13S1212Cit3-IgG synthetic peptides constructed based on HCs were significant (AUC=0.770, 0.687 and 0.698). 13S1212Cit3-IgM, 13S1210-IgG and 13S1212Cit3-IgG synthetic peptides had specificities of 94%, 94% and 98% and sensitivities of 60%, 43.3% and 41.7%, respectively (Fig. 2, Suppl. Table S4). We found that the

<table>
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<tr>
<th>Peptide name</th>
<th>Amino acid sequence</th>
<th>Corresponding amino acid sequence region and substitutions</th>
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<td>Arg7®Cit</td>
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Table I. Screening of the SLE specific peptides.

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<th>Phage added (pfu/ml)</th>
<th>Phage washed (pfu/ml)</th>
<th>Percentage enrichment (%)</th>
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<tbody>
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</table>

Percentage enrichment of phage clones (%) = (eluted phages/added phages) ×100%.

Table II. Sequence of positive displayed and citrullinated peptides.
COPSLE had comparative sensitivity and specificity, and had an AUC of 0.83 ($p<0.0001$, sensitivity 73.3% and specificity 88.0% at cut-off 0.2613), indicating that combination of peptides increased diagnostic accuracy (Fig. 2, Suppl. Table S4). Comparison of COPSLE and 13S1210-IgM ($p=0.0046$), 13S1212Cit3-IgG ($p=0.0001$), and 13S1212Cit3-IgG ($p=0.0001$) in ROC curves showed a significant difference in AUCs, indicating that a combination of reactivity may provide a higher degree of accuracy than any component reactions alone.

**Correlation between peptides profile and clinical data**
Comparing sera reactivity by Venn diagrams showed that 80.0% of anti-Sm-, anti-dsDNA- and ACA IgG/IgM-negative (triple negative) SLE sera was detected as positive using the peptide panel. We identified 37 of 52 Sm single-negative, 35 of 44 ds-DNA single-negative, and 33 of 44 ACA single-negative SLE patients, as well as seven of eight Sm single-positive, nine of 16 ds-DNA single-positive, and 11 of 16 ACA single-positive SLE patients using the peptide panel. Percentages of single-, double- and triple-negative or triple-positive SLE sera with positive reactions with individual panel peptides are shown in Figure 3A and B. These data indicated that the combined peptide assay was suitable for detecting SLE that appeared to be Sm-, dsDNA- and ACA-negative in conventional tests. We identified COPSLE in both anti-Sm, dsDNA and ACA antibody-positive and antibody-negative SLE patients.

We further analysed the relationship of COPSLE with individual clinical examination indicators using Spearman rank correlation (Suppl. Table S5: distribution of 55 CEIs used in this study), and found that COPSLE significantly correlated with albumin (ALB) ($r=0.2623$; 95% CI 0.0013–0.4899, $p=0.0429$), and decreased complement component C3 ($r=-0.3887$; 95% CI -0.5899 to -0.142, $p=0.0021$), serum level of C3 ($r=0.2866$; 95% CI 0.0276–0.5095, $p=0.0264$), discoid lupus ($r=-0.2754$; 95% CI -0.5005 to -0.0154, $p=0.0332$), and platelet distribution width (PDW) ($r=0.2637$; 95% CI 0.0028–0.4909, $p=0.0418$).

**Validation of microarrays for RA diagnostic biomarkers**
Positive rates of 13S1204-IgM (51.67%) and 08S1204Cit-IgG (35.0%)...
for RA were significantly higher than for HCs (p=3.50E-06 and 0.0021) and SLE (p=0.005 and 0.0025) (Fig. 4A, B). Positive rates of 13S1204-IgM for RA were significantly higher than for 13S1204Cit-IgM (p=1.77189E-07). Positive rates of 08S1204Cit-IgG for RA were significantly higher than for 08S1204-IgG (p=0.039). ROC curve analysis of 13S1204-IgM and 08S1204Cit-IgG synthetic peptides based on HCs was significant for RA (AUC=0.708 and 0.625). 13S1204-IgM and 08S1204Cit-IgG synthetic peptides both had specificity of 90.0%, and moderate sensitivities of 51.7% and 35.0%, respectively. Multivariable logistic regression analysis showed that for RA the combination COPRA of 13S12104-IgM (OR 10.572; 95% CI 3.550–31.480, p<0.0001) and 08S1204Cit-IgG (OR 5.625; 95% CI 1.787–17.709, p=0.0032) was a potential biomarker for diagnosis of RA, and improved the diagnostic ratio of RA (AUC=0.781, sensitivity 71.7%, specificity 82.0%). Comparison of COPRA and 13S12104-IgM (p=0.0030), 08S1204Cit-IgG (p=0.0001) in ROC curves showed a significant difference in AUCs (Fig. 4C).

Multivariable logistic regression analysis showed the combination COP-SLE/RA (combination of peptides to discriminate between SLE and RA) of 13S1204-IgG (OR 0.364; 95% CI 0.157–0.842, p=0.0183), 13S1212Cit3-IgM (OR 3.923; 95% CI 1.705–9.025, p=0.0013) and 08S1204Cit-IgG (OR 0.199; 95% CI 0.070–0.571, p=0.0027) could be a biomarker to discriminate between SLE and RA with 81.7% sensitivity and 65% specificity with AUC 0.767 (Fig. 4D). Thus, a combination of reactivity may provide a higher degree of accuracy than any of the component reactions alone.

Discussion

SLE is a model autoimmune disease resulting from an abnormality of cellular and humoral immunity (22). More than 100 autoantibodies have been found in SLE patients (23), but autoantibodies with high specificity and sensitivity in SLE have not been discovered. In recent years, Phage display techniques have been widely used to screen targeting peptides in drug discovery and biomarker selection, and have been highly effective in discovering peptides with affinities to virtually any target (24–28) in thrombotic thrombocytopenic purpura (29), acute anterior uveitis (30) and other autoimmune ocular inflammatory disorders (31). Microarrays show a consistently 4–8-fold higher sensitivity than ELISA for detecting antigen-specific autoantibodies (32), and has already been successfully used in the identification of new autoantibodies in diseases including autoimmune hepatitis (33), rheumatoid arthritis (34), breast cancer (35), ovarian cancer (36), and muscle-invasive diseases (37). Microarrays have been used to identify antibody reactivity for diagnostic applications (38), recognition of kinase-substrate activity for drug development, and discovery of peptide cell adhesion for investigating cell-cell communications (39). Other than detection of autoantibodies already identified in SLE, an important application of microarrays is the identification of novel autoantibodies associated with disease pathogenesis (40, 41).

In this study, we demonstrated that peptides from immune screening of a phage display 12-peptide library identified specific antibodies to distinguish SLE patients from RA patients and HCs. The method required minimal training and used only small volumes of serum. In addition to its discriminatory ability, the technical simplicity of this profiling technology makes it attractive for clinical use. We profiled serum antibodies using a microarray containing 37 unique peptides containing citrullinated peptides, and confirmed 13S1212Cit3-
IgM, 13S1210-IgG and 13S1212Cit3-IgG as novel biomarkers for SLE, with positive rates of 60.0–41.7%, comparable to anti-CCP for diagnostic strategies in RA (42-44). Since citrullination is a post-translational modification occurring in inflammation and citrullinated proteins have been detected also in other inflammatory arthritides and in inflammatory conditions other than arthritis (polymyositis, inflammatory bowel disease and chronic tonsillitis). So a citrullinated peptide is not specific for SLE, and anti-citrullinated protein antibodies are used as clinical biomarkers in other diseases (45-47). Similar pathogenetic mechanisms to those seen in RA may be relevant in a subgroup of SLE cases with a phenotype dominated by arthritis (48). The highest AUC was observed for 13S1212Cit3-IgM in SLE and HCs (AUC = 0.77). The positive rates of 13S1212, 13S1212Cit1 and 13S1212Cit2 were lower than for 13S1212Cit3, while 13S1210Cit in turn was lower than for 13S1210. Our data indicated that the design for using microarrays to screen for serum reactivity must consider the position of citrulline. ROC curve analysis suggested that 13S1212Cit3-IgM could be a diagnostic biomarker for SLE with 60.0% sensitivity and 94.0% specificity. Multivariate logistic regression analyses and maximum likelihood ratio tests indicated that COPSLE (combination of 13S1212Cit3-IgM and 13S1210-IgG) increased the likelihood of diagnosis of SLE. Adding 13S1212Cit3-IgG to COPSLE did not increase the likelihood of diagnosis of SLE further (data not shown), indicating that 13S1212Cit3-IgM has little diagnostic value. The sensitivity of COPSLE in microarrays was 73.30%, with 88.00% specificity at a cut-off of 0.2613 with AUC 0.830, which is comparable with commercially available tests. Results with microarrays showed that, although most SLE sera that recognise two peptides are IgG, some antibodies specifically recognising 13S1212Cit3 are IgM, which may be produced by newly formed activated B cells as an indication of the ongoing autoimmune response or by unswitched B memory cells. We found that 37 of 52 Sm-negative, 35 of 44 dsDNA-negative, 33 of 44 ACA IgG/IgM-negative and 26 of 30 triple-negative sera (Sm, dsDNA and ACA negatives) were recognised by the peptide panel. This result indicated that the combined assay detected some false-negative or false-positive SLE samples and some patients can be considered serum-positive, even if the disease is in remission.

Biomarkers inevitably overlap with diagnostics, which, in contrast, are intended only to inform individuals about present rather than future health status. When predicting lupus flares and assessing their activity with serological tests, anti-dsDNA, anti-nucleosome antibodies, anti-ribosomal proteins (23, 49), and decreased complement levels (C3 and C4) (50-52) and ALB (53) are commonly used, and are routinely tested in some clinics for SLE patients. The novel biomarkers were compared with 55 clinical examination indicators for SLE used in a validation set for univariate logistic regression. SLE Disease Activity Index in patients’ clinical data was unavailable. We found that COPSLE was negatively associated with decreased C3 and discoid lupus, and positively with ALB, PDW and serum C3. COPSLE may be a potential marker for disease remission in SLE.

Similar to many other antibodies reported, antibody biomarkers are often present in several autoimmune diseases (18). 13S1212Cit3-IgM, 13S1210-IgG and 13S1212Cit3-IgG were not unique to SLE; they were also present in RA although at a lower rate. However, we found that the positive rates of 13S1204-IgM and 08S1204Cit-IgG in RA were significantly higher than in SLE patients or HCs. COPRA of 13S1204-IgM and 08S1204Cit-IgG improved the diagnostic ratio (AUC = 0.781, 71.7% sensitivity, 82.0% specificity). Our study was a proof-of-concept only, and needs to be validated using a larger sample size before the microarray is applied in a clinical setting. Further studies regarding these issues are required. Our study suggested that a citrullinated/non-citrullinated peptide panel was a valuable diagnostic marker of SLE, even for patients who were negative for anti-Sm, anti-ds-DNA and ACA.

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