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Author Contribution

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Data Availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Abstract

Background: IgE-mediated fish allergy has long been considered an umbrella term due to the high cross-reactivity of parvalbumin, the major fish allergen. Yet, clinical tolerance to certain fish highlights allergenicity differences. In this study, we sought to construct a fish allergenicity ladder and identify fish parvalbumin epitopes to improve the diagnosis of fish allergy.

Methods: Reported clinical history and the serum-specific IgE (sIgE) responses of 200 fish allergic patients were collected and analyzed, while the relative parvalbumin content in different fish were measured for the construction of fish allergenicity ladder. Double-blind placebo-controlled food challenge (DBPCFC) and open challenge against salmon, grass carp and grouper were performed in 58 selected patients for validation of the ladder. Epitope mapping was performed by peptide array against parvalbumins of salmon (both β-1 and β-2), cod, grouper, and grass carp with sera from fish allergic (n=11), partial fish tolerant (n=12), and complete fish tolerant (n=5) patients diagnosed based on oral food challenge outcome.

Results: The distribution pattern of clinical, sIgE and molecular data and their strong positive correlation led to the construction of a 4-step fish allergenicity ladder comprising: step 1 of the least allergenic fishes (tuna, halibut, salmon), steps 2 (cod) and 3 (herring and grouper) of moderately allergenic fishes to step 4 of highly allergenic fishes (catfish, grass carp and tilapia). Epitope mapping revealed one epitope from grouper parvalbumin (AA64-78) for diagnosing general fish allergy and one epitopic region from salmon parvalbumin (AA19-33) as biomarker of specific fish tolerance. Only epitope-specific IgE differentiated these patients but not sIgE to fish extract or parvalbumin.

Conclusion: The fish ladder and epitopes discovery can precisely differentiate fish-allergic and tolerant subjects and guide fish reintroduction by stepping up the ladder, which innovate fish allergy care in the next millennium.

Keywords

IgE-mediated fish allergy; food ladders; epitope mapping; clinical cross-reactivity; home introduction

Introduction

Fish is one of the "Big 8" food allergens, with IgE-mediated fish allergy affecting approximately 1% of the world population and a much higher prevalence in pediatric cohorts at up to 7%[1-3]. Patients with fish allergy suffer from symptoms ranging from mild skin rashes to life-threatening anaphylaxis. Conventionally, fish allergy has been considered an umbrella term, and patients who are diagnosed to be allergic to one kind of fish are often advised to avoid all fish. Clinical reactions to multiple fish species have been mainly attributed to the cross-reactive major fish allergen, parvalbumin [4] . However, there are also reports of mono-allergy to single fish while tolerating others. Our team neatly demonstrated that a significant proportion of patients are tolerant to salmon despite experiencing severe allergic reactions to grass carp at double-blind, placebo-controlled food challenges (DBPCFC) [5] . Selection of appropriate fish for IgE testing and dietary reintroduction remains a major clinical gap due to the wide range of fish species from evolutionary distant classes consumed worldwide.

Parvalbumin is a 12-kDa sarcoplasmic calcium-binding protein for muscle contraction. It accounts for up to 95% of sensitization in patients with IgE-mediated fish allergy [6,7] . The capability of recombinant carp parvalbumin to induce the release of histamine from basophils of patients allergic to fish has long been reported [8] . Its high allergenicity (the ability to stimulate allergic reactions) can be attributed to its supreme stability even after cooking at high temperatures[9] . The activity of parvalbumin was also retained after treatment of denaturing agents, meaning that its allergenicity depends primarily on its amino acid sequence instead of higher-level structures. Parvalbumin has been shown to be involved in fast muscle relaxation, in which parvalbumin level and relaxation speed of muscles are closely and positively correlated[10] . It can mainly be found in fast-twitching white muscles, while endurable dark muscles in fish contain less parvalbumin. Therefore, migratory fish, such as tuna, have comparatively more dark muscle and hence less parvalbumin than sedentary fish, which have more white muscle instead [11] .

Differences in the protein levels of parvalbumin correlated with the difference in IgE reactivity to fish[12] . Clinical studies have indicated that sera of fish-allergic patients were more reactive to white muscle extract than to dark muscle extract, in which parvalbumin level was four to eight times lower [13] . However, a comprehensive large-scale study detailing the clinical reactions to multiple fish species, analysis of serological IgE reactivity and their correlation to the parvalbumin level of the corresponding fish, and the identification

of fish-specific IgE-binding epitopes for clinical cross-reactivity prediction is lacking. Herein, we report the clinical results of 200 fish-allergic subjects (specific IgE distribution, self-reported reactions to fishes, and DBPCFC data) and molecular analysis (parvalbumin levels determined by both protein and transcriptomic approaches and IgE reactivity of recombinant parvalbumins) for the construction of fish allergenicity ladder, as well as IgE-binding epitopes for clinical cross-reactivity prediction. Such ladder and the epitope results can stratify fish-allergic patients and the allergenicity difference of fishes to direct the selection of fishes for improving diagnosis and the gradual reintroduction of fishes in patients with IgE-mediated fish allergy.

Materials and Methods

Participant Population and History Collection

The study population comprised 200 physician-diagnosed fish-allergic patients recruited from five regional hospitals in Hong Kong (Prince of Wales Hospital, Queen Mary Hospital, Queen Elizabeth Hospital, Yan Chai Hospital and Princess Margaret Hospital) from 2019 to 2023. Subjects were recruited when they had a convincing history of immediate fish allergy as defined by immediate allergic symptoms within 2 hours following fish ingestion within two years before recruitment. Demographic data of the study population as well as their clinical history was collected with a standardized questionnaire. All participants were asked to indicate all fish species they had tried, including those eliciting an allergic response and tolerated without any symptoms, as well as their consumption pattern of fish in the past two years. Participants and/or their legal guardians gave written informed consent. Ethical approvals were obtained from the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee (reference 2019.612)

Allergy Assessment

Skin prick tests were performed using fish mix (containing cod, halibut and flounder) and salmon extracts (ALK-Abelló) according to the manufacturer's instructions with histamine and normal saline as controls. A prototype ImmunoCAP Grass carp test for research use only was developed as described previously [14] and specific IgE (sIgE) levels against nine fish extract ImmunoCAP tests (f3 cod, f40 tuna, f41 salmon, f205 herring, f303 halibut, f369 catfish, f410 grouper, f414 tilapia and grass carp), as well as fish parvalbumins (f355 rCyp c 1 and f426 rGad c 1) were measured on a Phadia 200 system. DBPCFCs with grass carp and salmon were performed in selected subjects as previously described, while a subgroup of patients (n = 9) also underwent open-labeled food challenges with grouper. The severity of allergic symptoms to fish was calculated based on the Ordinal Food Allergy Severity Score (oFASS)[15] . Briefly, grade 1 includes reactions restricted to oral cavity. Grades 2 to 5 may include oral symptoms but with other target organs affected, by which grades 2 and 3 reactions involve skin, eye/nose and/or digestive system. Reactions involving the larynx and/or bronchi are considered grade 4 reactions while grade 5 reactions would involve the cardiovascular and/or nervous systems.

Preparation of Recombinant Parvalbumins and ELISA

Protein sequences of parvalbumin from tuna (*Thunnus albacares* , Accession# CAQ72967), salmon (*Salmo salar* , β-1, Accession# CAA66403.1 and β-2, Accession# Q91483.3), cod (*Gadus morhua* , Accession# AAK63089.1), grouper (*Epinephelus lanceolatus* , Accession# XP_033500493.1), catfish (*Ictalurus punctatus* , Accession # AA025757.1), grass carp (*Ctenopharyngodon idella* , Accession# QCY53440.1) and tilapia (*Oreochromis mossambicus* , Accession #AAZ52553.1) were obtained from the NCBI database and reversed translated by MEGA X for cloning into His-tag expression vector pET30(A)+. His-tagged recombinant parvalbumins were then expressed in*Escherichia coli* BL21 (DE3) by culturing in MagicMedia (Invitrogen, Carlsbad, CA, USA) following the routine protocol in our laboratory[16-18] . Allergens were then purified using the HisPur cobalt spin columns (Thermo Fisher Scientific, Rockford, IL, USA) as per manufacturer's instructions. The concentration and purity of purified recombinant allergens were determined using the NanoDrop OneC spectrophotometer and SDS-PAGE, respectively. Purified recombinant parvalbumins were then coated onto MaxiSorp microtiter plates and incubated against diluted sera (1:10) from 37 fish-sensitized subjects for ELISA following our standard protocol as previously described [19] .

Parvalbumin Content and Expression Analysis

The relative content of fish parvalbumin was analyzed through both protein- and transcriptome-based approaches. For the protein approach, fresh fishes including tuna, salmon, halibut, cod, grouper, catfish, grass carp, and tilapia were purchased from local wet market. Genus/species of the fishes were confirmed by DNA barcoding upon DNA extraction with QIAamp DNA mini kit (Qiagen) and PCR with specific primers: Fish-F1: 5'-TCTCAACCAACCATAAAGACATTGG-3' and Fish-R1: 5'-TATACTTCTGGGTGCCCAAAGAATCA-3'; Fish-F2: 5'-CATCCTACCTGTGGCAATCAC-3'[20] . One milligram of raw fish meat was manually homogenized in 10mL ice-cold phosphate-buffered saline (PBS) until a smooth paste was achieved [21] . Protein was then extracted overnight at 4°C with constant stirring. The protein extract was centrifuged and supernatant was filter-sterilized through a 0.22 μm polyethersulfone membrane. The fish protein extracts analyzed fresh upon extraction. Ten microliters of fish extracts were then separated on a 13.5% SDS-PAGE gel according to their molecular weights using a Mini PROTEAN SDS-PAGE system (Bio-Rad). Protein bands were stained with SimplyBlue SafeStain (Thermo Fisher). Densitometry analysis was then performed to determine the relative band intensity of parvalbumin on SDS-PAGE by Image Lab (Bio-Rad) using salmon parvalbumin as the reference band (i.e. band intensity = 1). Four pieces of fish meat were randomly picked for protein extraction and resolved on separate SDS-PAGE to ensure reproducibility of parvalbumin level analysis. While in transcriptome-based approach, we processed publicly available RNA-seq data of fish meat, ensuring quality, and de novo assembly with Trinity[22]. Aligning transcripts with known allergens from AllergenOnline [23] via blast method identified homologous parvalbumin transcripts.

Mapping of IgE-binding Epitopes on Parvalbumin

Mapping of linear IgE-binding epitopes on parvalbumin was performed by PEPperPRINT. Epitope mapping was based on peptides of the sequences of parvalbumin from salmon (β-1, Accession# CAA66403.1 and β-2, Accession# Q91483.3), cod (Gad m 1, Accession# AAK63089.1), grouper (Accession# XP_033500493.1) and grass carp (Accession# QCY53440.1). The peptides ranged from 12 to 15 amino acids in length, offset by three amino acid residuals and printed in duplicate and framed by additional HA (YPYDVPDYAG, 60 spots) control peptides on microarray. Peptide microarray was pre-stained with the secondary antibodies in incubation buffer to determine background interactions that could interfere with the main assays. Incubation of further peptide microarrays with 29 serum samples (one pool of non-atopic control sample and 28 subjects who completed food challenge to salmon, grouper or cod, and grass carp) at successive dilutions of 1:20 was followed by staining with the anti-human IgE secondary antibody and read-out with an Innopsys InnoScan 710-IR Microarray Scanner. The additional HA peptides framing the peptide microarrays were subsequently stained with the control antibody as internal quality control to confirm assay performance and peptide microarray integrity.

Quantification of spot intensities and peptide annotation were based on 16-bit gray scale tiff files that exhibit a higher dynamic range than the 24-bit colorized tiff files shown in or provided with this report. Microarray image analysis was done with Mapix. A software algorithm breaks down fluorescence intensities of each spot into raw, foreground and background signal, and takes into account any flagging of artifacts by assigning the values -100 (artifact), 0 (standard) or 100 (very clear) to individual peptide spots. Based on artifact-corrected foreground median intensities, intensity maps were generated and interactions in the peptide maps highlighted by an intensity color code with green for high and white for low spot intensities. We tolerated a maximum spot-to-spot deviation of 40%, otherwise the corresponding intensity value was zeroed. This can be bypassed by manual flagging of peptides as "Artifact" or "Valid".

Data Analysis

Statistical analyses were performed by GraphPad Prism 8. Comparison of general characteristics of sensitized and non-sensitized subjects was evaluated by chi-square test, t test or Mann-Whitney U test where appropriate. Differences in sIgE among different fishes and components were compared using Friedman's ANOVA post-hoc test. Correlation analysis was performed with Pearson's correlation test. ED10 values

for salmon and grass carp (doses at which an allergic reaction would be elicited in 10% of the population in oral food challenge) were calculated as described [24] . The survival package 29 fin R30 was used for interval-censoring survival analysis. 95%CIs for the ED10 values were calculated by taking the 2.5^{th} and 97.5^{th} percentiles of the bootstrap distribution of ED10 values generated using 10,000 bootstrap samples. IgE-binding epitopes were defined for peptides with average spot signals (1) [?]1000 a.u., (2) > 3-fold of the signal from anti-human IgE secondary antibody, (3) > 3-fold of the signal from control serum sample, and (4) significantly higher signal intensity of allergic samples than tolerant samples (i.e. tolerated salmon or tolerated all fishes in food challenge) based on 1% false discovery rate (FDR) in Mann-Whitney test. Protein sequence alignment was performed on Clustal Omega [25] . A p-value of <.05 was considered statistically significant.

Results

Oral Food Challenge to Support the Fish Allergenicity Ladder

Based on the pattern of self-reported reaction and distribution of sIgE levels to different fishes (reactivities) and their positive correlation, we proposed that the analyzed fishes presented a ladder of allergenicity difference, from step 1 of the least allergenic fishes (tuna, halibut, salmon), steps 2 (cod) and 3 (herring and grouper) of moderately allergenic fishes to step 4 of highly allergenic fishes (catfish, grass carp and tilapia) (Fig 2). In an attempt to validate such a ladder, we analyzed results from 58 subjects who underwent DBPCFC with both salmon and grass carp. 48/58 (82.8%) subjects failed grass carp DBPCFC while only 19/58 (32.8%) failed salmon DBPCFC (Table S1). Among the 48 subjects who failed grass carp DBPCFC, 60.4% tolerated salmon. No salmon-tolerant subjects reacted to grass carp. ED10 (dose at which an allergic reaction would be elicited in 10% of the population in oral food challenge) was also remarkably higher for the salmon challenge at 10.7g (fish meat, 95%CI 0 – 18.6g) compared to grass carp at only 0.66g (95%CI 0 - 1.04g). On the other hand, we also selected nine subjects, three each from fish-tolerant (no reaction to both fishes), partially tolerant (tolerated salmon but reacted to grass carp) and fish-allergic (reacted to both fishes) groups to undergo open challenge with grouper (Table S1). None of the fish-tolerant subjects reacted to grouper, while all subjects in the partial tolerant and allergic groups failed grouper challenge. Although no remarkable difference in oFASS (i.e. severity of allergic symptoms) was detected, it is noted that 4/6 patients reacted at lower doses of grass carp but higher doses of grouper (mean eliciting doses at 24.3g and 32.0g fish meat, respectively), although the difference did not reach statistical significance with a small cohort size. These results further support the clinical need and relevance of the fish allergenicity ladder.

Comparison of Parvalbumin Reactivity and Levels in Different Fishes

Considering the strong correlation between fish extract-sIgE and parvalbumin-sIgE levels, we next examined if such allergenicity differences in fishes are attributed by (1) differences in IgE binding capacity of different fish parvalbumins, or (2) differences in parvalbumin levels in different fishes. Firstly, 37 serum samples from the sensitized group were assayed for IgE reactivity against purified recombinant parvalbumins from tuna, salmon (β-1 and β-2), cod, grouper, catfish, grass carp and tilapia (Fig 3A). Our data showed that IgE binding did not increase along the allergenicity ladder (Fig 3B). No statistical difference was detected among the tested parvalbumins even when comparing between tuna and tilapia, despite an 11-fold lower tuna-sIgE than tilapia-sIgE. Interestingly, salmon parvalbumin β-1 had remarkably lower IgE binding comparing to all parvalbumins except for tuna parvalbumin while grass carp parvalbumin had significantly stronger IgE binding than parvalbumins from tuna, salmon β-1, cod and tilapia. These results suggested that the difference in allergenicity is not due to IgE binding capacity of parvalbumins.

We next compared parvalbumin levels in the extracted fish protein by SDS-PAGE and densitometry analysis. Parvalbumin appeared as a 9-12 kDa protein in all fish extracts, while two parvalbumin isoforms could be visualized in halibut, grass carp and tilapia (Fig 3C). With reference to salmon parvalbumin (relative intensity = 1), the relative parvalbumin content showed no statistical difference among tuna, halibut and salmon, as well as among catfish, grass carp and tilapia (Fig 3D). Parvalbumin content was significantly higher in cod

comparing to salmon, halibut, and tuna, as well as in grouper comparing to cod. It is noted that parvalbumin levels in catfish, grass carp (isoform 2), and tilapia (isoform 2) were significantly higher than that in all other tested fishes. Importantly, the relative levels of parvalbumin positively correlated with the sIgE reactivity of the respective fish (r = 0.925, p =.001) and incident of self-reported allergic reactions (r = 0.89, p =.004). We also further validated the expression of parvalbumins by a transcriptomic approach. The transcriptomic expression level of these parvalbumins was similar to the results from protein-based analysis (Fig 3E). Yet, it is noted that the expression of GAPDH and aldolase were remarkably higher in halibut, yellowfin tuna and salmon comparing to the freshwater fishes, cod and grouper. These results suggested that the difference in allergenicity is due to the amount of parvalbumin in fish muscle.

IgE-binding Regions for Cross-reactivity and Fish-specific Tolerance Prediction

To determine whether sensitization to specific parvalbumin epitopes could predict cross-reactivity and clinical reactivity along the allergenicity ladder, epitope mapping was performed against parvalbumins of salmon (both β-1 and β-2), cod, grouper, and grass carp with sera from fish allergic (n=11), partial fish tolerant (n=12), and complete fish tolerant (n=5) patients based on oral food challenge outcome. Highly stringent criteria of signal intensity [?]1000 a.u., control serum, and significantly higher signal intensity of allergic samples than tolerant samples (i.e. tolerated salmon or tolerated all fishes in food challenge) based on 1% false discovery rate (FDR) were adopted to identify specific IgE-binding epitopes that can differentiate allergy and tolerance to specific fishes. Comparison between grass carp allergic and tolerant subjects revealed AA64-78 (Epi_c_64-78, LKLFLQNFSAGARAL) from grouper parvalbumin as IgE-binding epitope (Fig 4A&B, Fig S1). This epitope strongly and specifically reacted with 21/23 (91.3%, >500 fluorescence intensity) fish allergic subjects and none of the tolerant patients, and also represented the only parvalbumin epitope with such diagnostic accuracy. This epitope thus denoted a cross-reactivity biomarker to identify "general" fish allergy.

We also studied allergic and partially tolerant individuals to identify epitopes and predict fish-specific tolerance. AA19-48 of salmon parvalbumin β-1 (Sal_s_β1_19-48; CKAADTFSFKTFFHTIGFASKSADDVKKAF), AA23-37 of salmon parvalbumin β-2 (Sal_s_β2_23-37; DSFNHKAFFAKVGLA), AA34-48 of cod parvalbumin AA34-48 (Gad_m_34-48; CGLSGKSADDIKKAF) and AA16-36 of grouper parvalbumin (Epi_c_16-36; IAGC-SAADSFDHKKFFKACGM) fulfilled the criteria as IgE-binding epitopes and to further identify salmon tolerance (Fig 4C-G and Fig S2-5). Among these regions, AA19-33 of salmon parvalbumin β-1 (Sal_s_β1_19-33) was exclusively bound by IgE of allergic patients at high intensity, but not by partially or completely fish tolerant individuals (Fig S2). This epitope thus represented a novel biomarker to differentiate salmon-specific tolerance in fish (grass carp) allergic patients. No major IgE-binding sites could be detected from grass carp parvalbumin based on our criteria (Figure S5). We could not detect significant correlation between sIgE level and epitope signal intensity, meaning that IgE binding to these epitopes were independent of patients' sIgE levels to the respective fish extract (Fig S6). Most importantly, neither fish-specific nor parvalbumin-specific IgE levels distinguished between allergic and partially tolerant patients, or between salmon allergic and tolerant individuals (p > .05 and q > .16) but only the identified epitopes (Fig S7).

Discussion

Fish allergy is common, often life-long, and has a major impact on the pediatric population and their families. Fish allergy has long been an umbrella term, while clinical management of fish allergy is particularly complex due to the huge diversity of edible fish but extensive cross-reactivity, and the presence of multiple fish allergens with sensitization dependent on culture, dietary habits and cooking methods. Precision diagnosis for specific fish allergy and tolerance has major implications for proper patient labeling, reducing unnecessary food avoidance and reintroducing fish into patients' diets. Our study is the first to present a fish allergenicity ladder based on both clinical and molecular data, and validated by oral food challenges. We also, for the first time, identified IgE-binding epitopes of parvalbumins from salmon, cod, grouper, and grass carp with patients diagnosed by DBPCFC for predicting clinical cross-reactivity and fish-specific allergy.

The fish allergenicity ladder presented in this study is robustly constructed based on both the sIgE levels of

a large cohort of 166 physician-diagnosed fish-allergic and sensitized subjects against nine fishes, as well as the patients' clinical reactivity against a vast panel of marine and freshwater fishes. Grass carp and salmon were the two most common fishes first introduced as solid foods (in congee) in our traditional Chinese diet, and the difference in the percentage of reported tolerance was obvious (40.2% for salmon and 7.5% for grass carp). Likely subsequent to the allergic episode to these fishes followed by a positive SPT and/or sIgE test during clinical check-ups, a majority of our fish-sensitized subjects avoided (i.e. naïve) other fishes like tuna, halibut, cod, grouper, catfish and tilapia that were usually introduced later in our pediatric cohort. Interestingly, the pattern of allergy and tolerance to these fishes was still clear and strongly correlated with the sIgE levels to the respective fishes. Similar tolerance pattern was also reported in Singaporean fish allergic subjects, by which 75.9% fish allergic children tolerated some fish species with salmon (37.0%), tuna (24.1%) and cod (22.2%) being the leading tolerated fishes [26] . Our oral food challenge results from 58 fish-sensitized subjects also conformed well with our analysis on patients' self-reported history. Apart from a much higher incidence of grass carp than salmon allergy and the high proportion of partial tolerance to salmon in grass carp allergic patients (60.4%), ED10 to grass carp was also almost 35-fold lower comparing to that of salmon. Similar DBPCFC results were also reported, by which 6% of subjects reacted to salmon only comparing to 20% who reacted to cod only, and 11% reacted to both cod and mackerel comparing to none reacted to salmon and mackerel [27] . In subjects with fish allergy, differences in the specificity of allergenicity is a critical consideration in the process of fish reintroduction.

Our findings on the high parvalbumin to extract ratio and strong correlation between sIgE to fish extract and parvalbumins suggested that parvalbumin is the major allergen in our cohort as in most studied populations [7,28] . We showed that the relative level of parvalbumin increases along the allergenicity ladder constructed based on sIgE levels and clinical history. For instance, parvalbumin content increases from tuna, halibut, salmon, cod, grouper, catfish, grass carp and tilapia as validated by both proteomics and transcriptomic analysis. Such findings agreed with previous reports that large migratory fishes with more dark muscle have lesser parvalbumins for continuous swimming, while sedentary fishes have more white muscle and parvalbumin for short burst swimming [29] . Parvalbumin content was as low as <1mg/g in salmon and tuna while the difference can be 48 times comparing between splendid alfonsino and bigeye tuna. Our study here describes beyond such difference and further showed that fish parvalbumins present the same molecular allergenicity by testing eight recombinant parvalbumins from tuna, salmon, cod, grouper, and the freshwater fishes with 37 allergic samples. Our findings also demonstrated the strong and positive correlation of parvalbumin content with sIgE reactivity and also incident of self-reported history in allergic patients, which emphasize the clinical attribution of parvalbumin content to differential sensitivity to fishes. A case study of fish oral immunotherapy (OIT) with stepwise increase of parvalbumin from 1mg to 66mg over two years led to reduced parvalbumin-specific IgE and elevated level of IgG4[30] . While "conventional" fish OIT ingesting the same fish over years can put patient at risk of heavy metal toxicity and develop food anxiety, gradually introducing a wider spectrum of fishes along the allergenicity ladder can be an alternative form of fish OIT. Regular administration of food allergen along the ladder is likely to attain similar immune changes in OIT that assist to expand patients' diet and establish tolerance. For instance, milk and egg ladders that were originally outlined for managing non-IgE-mediated food allergy have been extended to the management of IgE-mediated allergies[31,32] . A handful of studies have now been published illustrating the safety and efficacy of milk and egg ladders that participants could tolerant more allergenic foods just after a year[33-39] . Even consuming baked goods regularly in step 1 of the food ladder also promoted tolerance[40,41] . There is increasing recognition that children with fish allergy can tolerate some fish species while lessons from egg and milk allergy inform the possible resolution of food allergy through a step-wise progression from extensively heated (low allergenicity) to less heated (high allergenicity) foods.

Fish ladder can thus embark on fish allergy management through facilitating dietary expansion and encouraging the resolution of fish allergy.

Based on such fish allergenicity ladder, we further extended our analysis for IgE-binding epitopes with the specific emphasis to predict clinical cross-reactivity and fish-specific allergy/tolerance. To our knowledge, this is the first study to identify IgE-binding epitopes of parvalbumins from four different fish species of

differential allergenicity at each step (salmon, cod, grouper, and grass carp) with sizable samples from fish allergic (n=11), partial fish tolerant (n=12) and complete fish tolerant (n=5) subjects diagnosed by DBPCFC, the gold standard of food allergy diagnosis. We identified Epi_c_64-78 from grouper parvalbumin as major cross-reactive IgE-binding epitopes to identify "general" fish allergy. This epitope strongly and specifically reacted with grass carp and grouper allergic subjects but none of the tolerant patients, and overlaps with the previously described regions in salmon [42] . This epitope sequence shared an average sequence homology of 61.2% with other tested parvalbumins, and 69.7% similarity with the homologous sequence in grass carp parvalbumin. We also, for the first time, identified an IgE-binding region at Sal_s_β1_19-33 that stand as a novel biomarker to differentiate salmon tolerance among subjects allergic to grass carp and/or grouper. We also importantly showed that such prediction could not be achieved by fish- or parvalbumin-specific IgE levels of these individuals but only their sIgE reactivity to this epitope region. Predicting such tolerance in fish allergic patients is important when considering the beneficial effects of fish in young children. For instance, oily fish such as salmon is rich in omega-3 fatty acids that can protect against heart disease and support neuronal growth and brain development [43] . These two epitopes will be of great value for precision diagnosis of fish allergy, then better advise on fish reintroduction in allergic patients. Further testing and validation of the epitopes identified in this study in larger and multinational cohorts of fish allergic patients with known fish-specific allergy and tolerance are warranted.

We are aware that the present study comprises only Chinese patients, and analyzed parvalbumin content and fishes available on the ImmunoCAP system only. The applicability of the allergenicity ladder in other populations with different dietary habits and sensitization to other important fish allergens have yet to be considered. Epitope validation and extending this fish allergenicity ladder to a wider spectrum of fishes, and consider also different cooking methods and time for a more comprehensive ladder will the next steps to move fish allergy care into the next millennium and improve the quality of life of fish allergic individuals and their families. It is worth mentioning that this is the first attempt to compare the relative expression of fish allergens with a transcriptomic approach. Interestingly, the expression of aldolase has a "reverse" pattern contrary to parvalbumin, by which the relative expression of aldolase peaks in salmon, followed by yellowfin tuna, halibut and grouper, and appears low in freshwater fishes and cod. Aldolase, enolase and collagen are important fish allergens and mono-sensitization to these allergens has been well-documented[6,44,45] . Unlike sera from patients with parvalbumin-specific allergy that reacted strongly with fishes of high parvalbumin content and weakly with fishes of low parvalbumin content, those with collagen-specific allergy reacted similarly to all 22 species of fishes despite the varying levels of parvalbumin[46] . It is therefore important to test for the major sensitizing allergen in patients implicated with fish allergy, followed by screening epitope-specific IgE for possible fish tolerance under the framework of precision diagnosis. While parvalbumin accounts for >80% sensitization in fish allergic individuals, most patients can rely on this propose fish ladder based on parvalbumin levels. Our two identified epitopes and fish allergenicity ladder are clinically useful in these patients with parvalbumin-specific allergy for selecting fish in each step of the ladder for further IgE testing, to inform which step of the fish ladder to start with during reintroduction, and to guide fish reintroduction by gradually stepping up the ladder from tolerant fishes or low allergenicity fishes to increase the threshold dose to fish parvalbumin and achieve remission of fish allergy.

In summary, the parvalbumin epitopes and fish allergenicity ladder presented in this study can serve as a new compass to guide IgE testing and fish reintroduction. These can benefit a majority of fish allergic patients considering the role of parvalbumin as a major fish allergen across different geographical populations. Particularly in the era of telehealth, fish ladder guiding allergenic food reintroduction at home is convenient, and can greatly reduce burden on clinical care and ease financial strain.

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Tables Table 1 Demographics of recruited participants

	Sensitized	**Non-sensitized**	**p-value**
Number	166	34	
Age at recruitment; median (range); years	4.89 (0.8-33)	5.6 (1.1-21.5)	0.56
Males (n, %)	113, 68%	23, 68%	0.96
Asthma (n,%)	56, 33%	12, 35%	0.85
Atopic dermatitis (n, %)	155, 93%	29, 85%	0.16
Allergic rhinitis (n, %)	83, 50%	14, 41%	0.45
Age of onset; median (range); months	9 (3-60)	12 (5-48)	0.23

	Sensitized		**Non-sensitized**	**p-value**
Anaphylaxis	23, 14%		3, 9%	0.58
Total IgE; median (range); kU/L	663 (0.57-5000)		189 (0.55-1289)	**<0.0001**
SPT fish mix; median (range); mm	5.0 (0-17.0)		0 (0-3.0)	**<0.0001**
SPT salmon; median (range); mm	4.0 (0-17.0)		0 (0-3.5)	**<0.0001**
Self-reported causative fish				
1st - Grass carp (n, %)	73, 44%	1st - Salmon (n, %)	15, 44%	
2nd - Salmon (n, %)	67, 40%	2nd - Grass carp (n, %)	10, 29%	
3rd - Grouper (n, %)	51, 31%	3rd - Cod (n, %)	8, 24%	
Any marine	112, 67%		26, 76%	0.42
Any freshwater	98, 59%		12, 35%	0.01
Self-reported tolerant fish				
1st - Salmon (n, %)	45, 27%	1st - Salmon (n, %)	14, 41%	
2nd - Tuna (n, %)	14, 8%	2nd - Grass carp (n, %)	9, 26%	
3rd - Halibut (n, %)	8, 5%	3rd - Grouper (n, %)	8, 24%	
Any marine	56, 43%		21, 62%	0.003
Any freshwater	8, 5%		13, 38%	**<0.0001**
Any one fish	63, 38%		25, 74%	0.0002

Table 2 Distribution of sIgE and correlation analysis

ImmunoCAP	**Median, kUA/L (IQR)**	**[?]0.35 kUA/L (n, %)**	**Class 0 (n, %)**	**Class 1 (n, %)**	**Class 2 (n**
Tuna	0.7 (0.2 - 2.2)	112 (67.5%)	54 (32.5%)	28 (16.9%)	52 (31.3%)
Halibut	1.4 (0.3 - 3.9)	120 (72.2%)	46 (27.7%)	21 (12.7%)	51 (30.7%)
Salmon	1.3 (0.4 - 5.5)	128 (77.1%)	38 (22.9%)	17 (10.2%)	59 (35.5%)
Cod	1.6 (0.4 - 6.4)	126 (75.9%)	40 (24.1%)	13 (7.8%)	55 (33.1%)
Herring	2.7 (0.6 - 8.6)	136 (81.9%)	30 (18.1%)	14 (8.4%)	47 (28.3%)
Grouper	2.4 (0.7 - 10.0)	137 (82.5%)	29 (17.5%)	14 (8.4%)	48 (28.9%)
Catfish	4.8 (1.3 - 22.2)	156 (93.9%)	10 (6.0%)	15 (9.0%)	39 (23.5%)
Grass carp	4.9 (1.6 - 21.8)	158 (95.2%)	8 (4.8%)	15 (9.0%)	45 (27.1%)
Tilapia	5.9 (1.4 - 24.0)	159 (95.8%)	7 (4.2%)	13 (7.8%)	44 (25.3%)
rCyp c 1	6.4 (1.4 -27.4)	157 (94.5%)	9 (5.4%)	9 (5.4%)	43 (25.9%)
rGad c 1	4.2 (0.7 - 14.9)	144 (86.7%)	22 (13.3%)	17 (10.2%)	35 (21.1%)

Figures

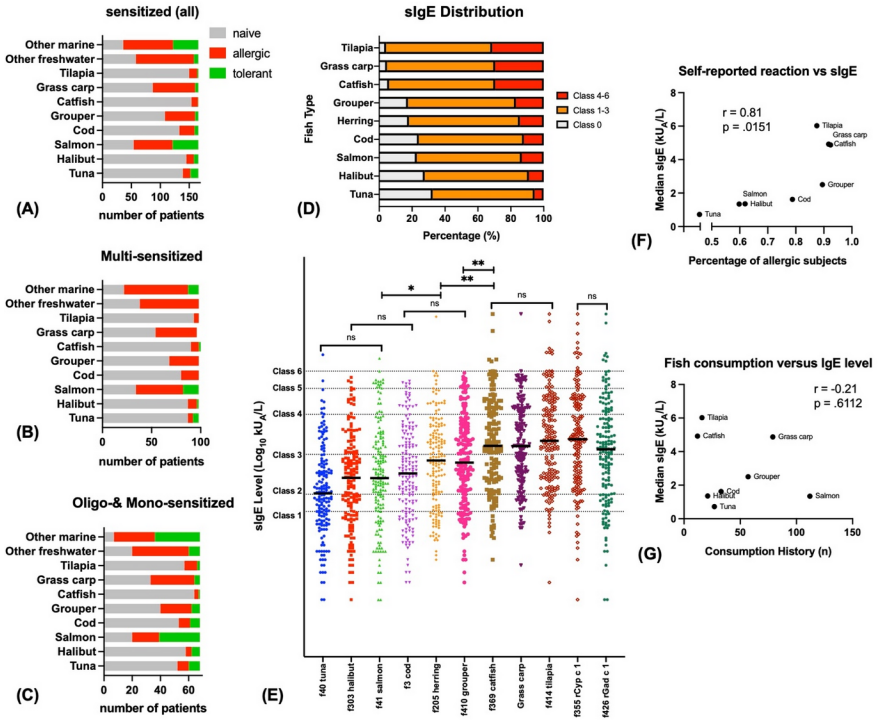


Figure 1 Self-reported history and sIgE distribution of sensitized fish subjects . Self reported history of (A) sensitized fish subjects (n=166), (B) multi-sensitized subjects (n=98) who tested positive to all 11 ImmunoCAP tests, (C) oligo- & mono-sensitized subjects (n=68) who were sIgE positive to [?]1 ImmunoCAP test. All subjects were naïve to herring and data was omitted. (D) sIgE class distribution and (E) sIgE levels to fish extracts and parvalbumins of sensitized subjects. Statistical comparison was performed by Friedman's ANOVA post-hoc test. sIgE class: Class 0: < 0.35 kUA/L; Class 1: 0.35-0.7 kUA/L; Class 2: 0.71-3.5 kUA/L; Class 3: 3.51-17.5 kUA/L; Class 4: 17.6-50 kUA/L; Class 5: 50-100 kUA/L; Class 6: >100 kUA/L. Note that no difference in sIgE between (1) tuna, halibut and salmon; (2) cod, herring and grouper; (3) catfish, grass carp and tilapia; and (4) halibut, salmon and cod were detected (ns). Herring-sIgE was statistically higher than both halibut- and salmon-sIgE (*p<.05), while catfish-sIgE was also statistically higher than both herring- and grouper-sIgE (**p<.01). Correlation between sIgE and (F) self-reported reactions (percentage of subjects with history of allergic reaction) and (G) consumption history (number of subjects who reported consumption). Note the positive and significant correlation between sIgE level and incident of self-reported allergic reactions to the respective fish (r = 081, p = .0151) but not with the frequency of consumption to the specific fish (r = -0.21, p = .6112)

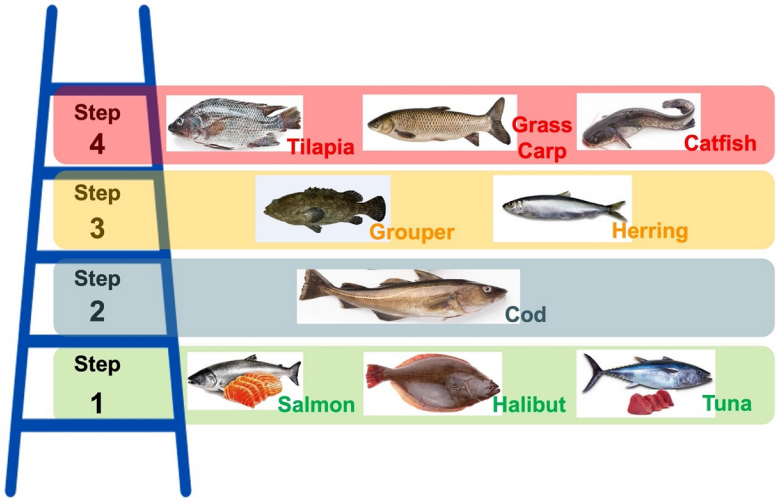
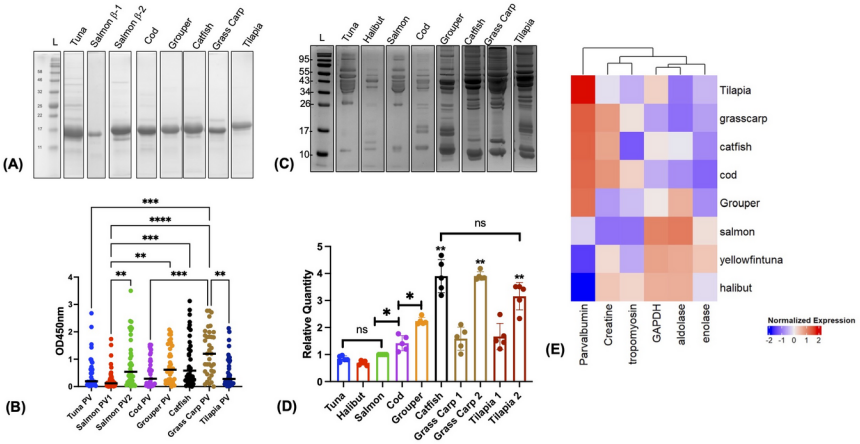


Figure 2 Fish allergenicity ladder. The ladder was constructed based on self-reported history of allergic reactions, sIgE reactivity and protein level of parvalbumin of the fishes. Step 1 refers to the fishes with lowest allergenicity, and allergenicity of the fishes step up along the ladder.



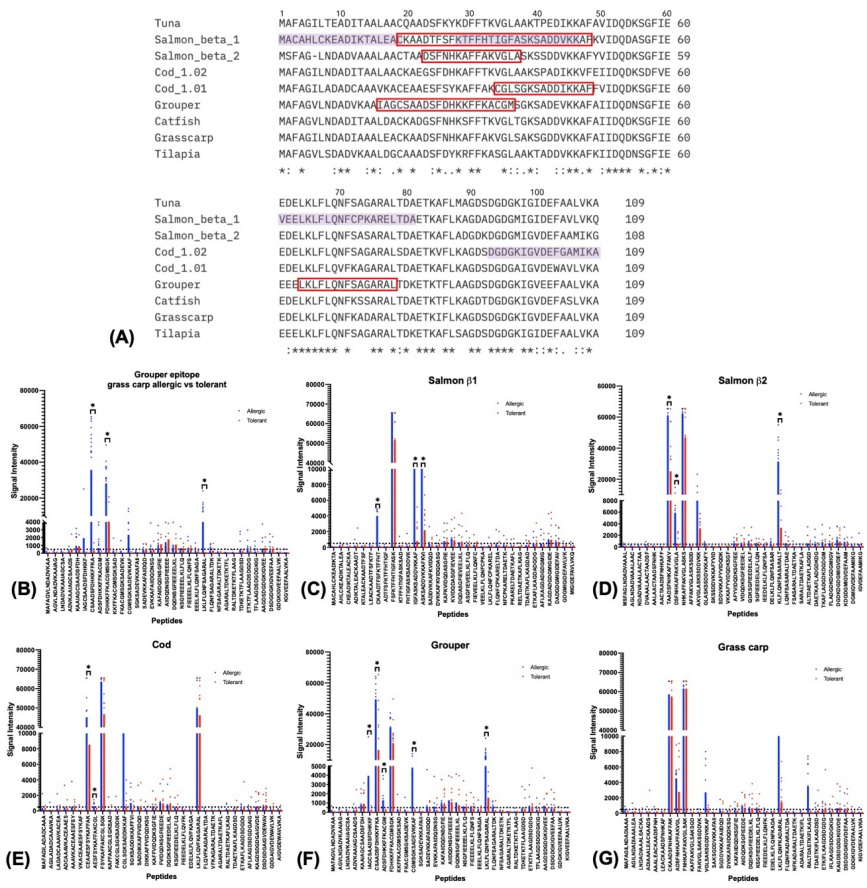


Fig 4 IgE-binding epitopes of fish parvalbumins. (A) Aligned protein sequences of fish parvalbumins showing major IgE-binding epitopes. Purple-shaded sequences are epitopes previously identified. Boxed sequences are IgE-binding regions identified in this study. (B) Signal intensity of the overlapping parvalbumin peptides of grouper compared between grass carp allergic and tolerant subjects. Signal intensity comparison between allergic and partial tolerant subjects of overlapping parvalbumin peptides of (C) salmon β-1, (D) salmon β-2, (E) cod, (F) grouper and (G) grass carp. Significant difference between allergic and partial tolerant individuals (*q-value <.05) on Mann-Whitney test are showed.