TechNotes

HyTest

Clinical and Research Area | Cardiac Markers

HyTest Proprietary IGFBP-4 Technology

Pregnancy-associated plasma protein-A (PAPP-A) is expressed in unstable atherosclerotic plaques and PAPP-A has been proposed to be a marker of unstable plaques. PAPP-A is a protase that cleaves insulin-like growth factor binding-protein-4 (IGFBP-4) and therefore increased concentrations of IGFBP-4-fragments in blood cir-culation could serve as a read out of increased PAPP-A activity in plaques. HyTest has developed two proprietary assays for measurement of N- and C-terminal IGFBP-4 fragments and preliminary results from clinical studies show that IGFBP-4 fragments are potential new markers for major acute cardiac event (MACE) prediction. This proprietary technology is now available for licensing.

Background

Atherosclerosis – plaque formation in the blood vessel walls – is a systemic and multifocal disease that starts early in life and usually decades are needed before disease eventually appears as a consequence of progressive obstruction or abrupt thrombotic occlusion. Atherosclerotic plaques are divided into two broad categories: stable and unstable (also called vulnerable).

Stable coronary disease may last for decades and remain asymptomatic. In contrast, unstable myocardial ischemia symptoms usually result from a thrombus caused by a disrupted atherosclerotic plaque in coronary artery. This event is called a myocardial infarction (MI).

Current diagnosing methods detect coronary stenosis accurately in severe cases but they are not able to reveal the underlying processes and therefore there is a need for accurate risk markers.

Plaque destabilization is a highly complex multifactorial process requiring proteinases that digest the extracellular matrix. One of these proteases that is thought to be involved in the plaque destabilization is dimeric form of Pregnancy Associated Plasma Protein A (dPAPP-A)-metalloproteinase and several studies show that expression of dPAPP-A is significantly increased in unstable atherosclerotic plaques.

Working Hypothesis

It has been shown that dPAPP-A is able to cleave IGFBP-4 thus forming N- and C-terminal fragments (NT- and CT-IGFBP-4) with molecular masses of 18 and 14 kDa, respectively.

It was concluded that dPAPP-A expressed in atherosclerotic plaques is an active protease able to cleave IGFBP-4 and local increased expression of dPAPP-A would increase N- and C-terminal IGFBP-4 fragments' concentrations in patients' blood thus indicating the level of plaque instability.

It is hence proposed that N- and C-terminal IGFBP-4 fragments can serve as new biomarkers for risk assessment of cardiac events and all-cause mortality in patients with myocardial ischemia.

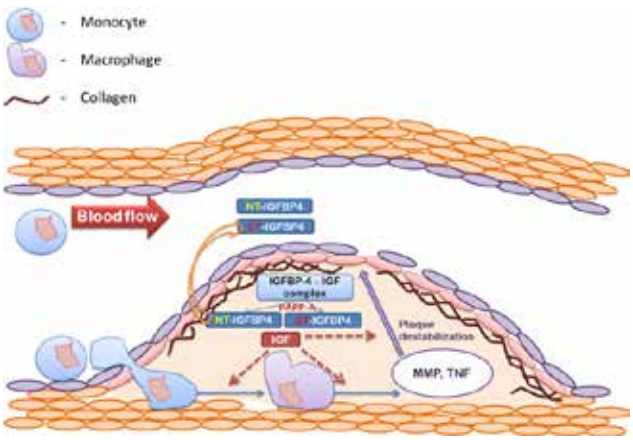

Figure 1. Schematic representation of NT- and CT-IGFBP-4 assays.

Materials

Monoclonal Antibodies

HyTest has developed monoclonal antibodies (MAbs) which are specific to novel epitopes formed by proteolytic cleavage of IGFBP-4 by PAPP-A. These MAbs recognize only N- or C-terminal fragments of IGFBP-4 and have no cross-reaction with intact (full-length) molecule.

MAb IBP3 is specific to novel epitope of NT-IGFBP-4 and MAb IBP163 recognizes novel epitope of CT-IGFBP-4.

Human recombinant NT-, CT-, and full-length IGFBP-4

Human recombinant NT- and CT-IGFBP-4 were used as calibrators for the corresponding immunoassays. It was also used in the studies demonstrating specificity of NT- and CT-IGFBP-4 immunoassays. Proteins were purified to >90% homogeneity by immunoaffinity chromatography (See Fig 2).

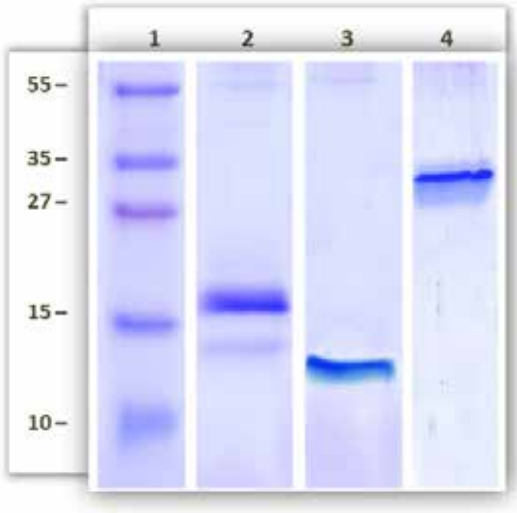

Figure 2. SDS-PAGE analysis of purified recombinant NT-, CT-, and full-length IGFBP-4.
Line 1 – molecular weight markers
Line 2 – recombinant NT-IGFBP-4
Line 3 – recombinant CT-IGFBP-4
Line 4 – recombinant full-length IGFBP-4

Recombinant proteins were analyzed using matrix-assisted laser desorption/ionization mass spectrometry and the N-terminus of CT-IGFBP-4 was confirmed using N-terminal sequencing.

Immunoassays

Monoclonal antibodies. MAbs IBP3 and IBP144 were used in the N-terminal IGFBP-4 fragment specific assay. IBP3 is specific to the N-terminal fragment (NT-IGFBP-4) and does not cross-react with the full length IGFBP-4 while IBP144 recognizes both. IBP3 was used as a capture antibody and IBP144 was used as a labeled antibody.

C-terminal fragment specific immunoassay utilized monoclonal antibodies IBP182 and IBP163 as capture and label antibodies, respectively. MAb IBP182 recognizes full-length IGFBP-4 and CT-IGFBP-4 while MAb IBP163 is specific to CT-IGFBP-4 and has no cross-reaction with full-length IGFBP-4.

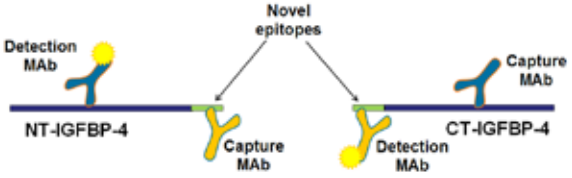

Figure 3. Schematic representation of NT- and CT-IGFBP-4 assays.

Immunoassay. Capture antibodies (20 mg/L in PBS, 0.1 mL per well) were incubated in 96-well immunoassay plates for 45 min at room temperature with constant shaking. After incubation plates were washed with 10 mM Tris-HCl (pH 7.8) buffer, supplemented with 0.15 M NaCl, 0.025% Tween 20 and 0.5 g/L NaN_3 (buffer A). After washing, 0.05 mL of analyzed plasma sample diluted 5-8 times with assay buffer (50 mM Tris-HCl buffer, pH 7.7, 9 g/L NaCl, 0.01% Tween 40, 0.5% BSA and 0.5 g/L NaN_3), and then 0.05 mL of detection antibodies (4 mg/L) were added to the plates. In all assays detection MAbs were labeled with stable Eu^{3+} chelate. Plates were incubated for 90 min at room temperature with constant shaking. After washing with buffer A the fluorescence of Eu^{3+} was measured in the presence of enhancement solution on a Victor 1420 multilabel counter (Wallac-Perkin Elmer). The fluorescence was expressed in counts per second (CPS).

NT-IGFBP-4 immunoassay with HyTest's recombinant human NT-IGFBP-4 is shown in Fig. 4. The analysis of the assay for NT-IGFBP-4 showed that the assay had no or very low (0.4%) cross-reaction with the full-length IGFBP-4 molecule. CT-IGFBP-4 immunoassay with recombinant human CT-IGFBP-4 used as a calibrator is presented also

in Fig. 4. CT-IGFBP-4 assay had very low (1.4%) cross-reaction with the full-length IGFBP-4.

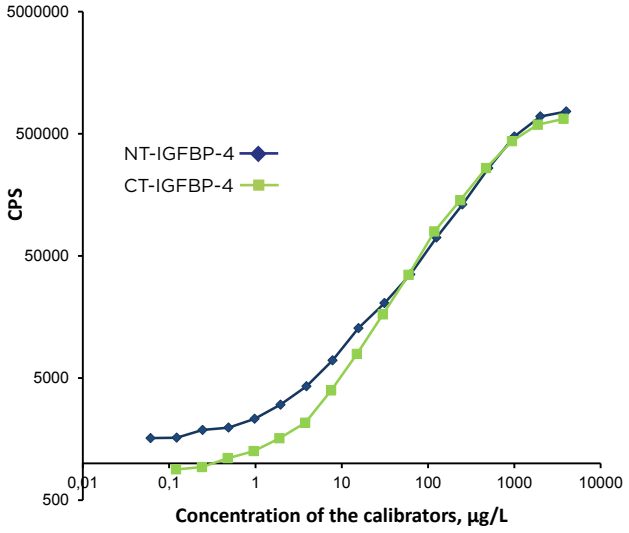

Figure 4. NT-IGFBP-4 immunoassay with HyTest's recombinant human NT-IGFBP4.

Assay validation

The limit of blank (LoB) was determined in accordance with Clinical and Laboratory Standards Institute (CLSI) requirements. The LoD represents the 95th percentile value from n ≥ 60 measurements of analyte-free samples over several independent series.

Linearity was assessed by serial dilution of 3 human serum samples. Each sample was diluted up to 1:100 with assay buffer in 4 consecutive steps. All dilutions were performed in triplicate and linearity was calculated separately for each measurement.

Recovery studies were performed by spiking pooled normal EDTA-plasma free samples with 2, 100, and 1000 µg/L of the calibrator.

Stability studies were performed using EDTA-plasma free samples spiked with 2, 40, and 600 µg/L of the recombinant material and samples were incubated at room temperature for 0, 1, and 2 hours whereafter they were analyzed using the immunoassays. Freeze/thaw stability of the analytes was tested using the same samples. IGFBP-4-fragment levels changed less than 10% in both types of stability tests.

Cross-reactivity of both assays was tested with recombinant IGFBP-5. Results of the validation of both assays are shown in Table below.

Characteristic	NT-IGFBP-4	CT-IGFBP-4
Limit of detection, µg/L	0.4	0.8
Linear range, µg/L	2.0 - 1000	12 - 500
Within-assay imprecision (CV), %		
Low concentration (2 µg/L)	4.6	7.4
Medium concentration (40 µg/L)	3.1	3.1
High concentration (600 µg/L)	3.7	6.1
Total imprecision (CV), %		
Low concentration (2 µg/L)	11.3	10
Medium concentration (40 µg/L)	6.2	8.9
High concentration (600 µg/L)	7.2	7.4
Recovery, %		
Low concentration (2 µg/L)	84	72
Medium concentration (100 µg/L)	99	79
High concentration (1000 µg/L)	82	69
Cross-reactivity, %		
NT-IGFBP-4	100	0.1
CT-IGFBP-4	0.1	100
IGFBP-4	0.4	1.4
IGFBP-5	0	0

Clinical studies

ACS Study. IGFBP-4 fragments were measured in EDTA-plasma samples of 180 patients with confirmed non-ST elevation acute coronary syndrome (ACS). The diagnosis of non-ST-elevation MI (non-STEMI) was based on presence of signs or symptoms of myocardial ischemia. Both NT- and CT-IGFBP-4 were shown to be strong predictors of MACE including MI and cardiac death in 6-months follow-up period (the values of area under the ROC curve were 0.86 and 0.81, respectively). It should be noted that most part of end-points occurred in 3-months period.

CT-IGFBP-4 *clinical validation*. CT-IGFBP-4 levels were measured in EDTA-plasma of 406 patients. The incidence of MACE as well as all-cause deaths (ACD) was recorded during one year follow-up period. The median plasma CT-IGFBP-4 concentration was 62 µg/L. During the follow-up, 59 (14.5%) patients met MACE end point and 43 (10.6%) met ACD end point. CT-IGFBP-4 as a predictor of MACE and ACD showed an area under the ROC curve of 0.66 and 0.75, respectively.

Plasma CT-IGFBP-4 levels of >90 µg/L were 50% sensitive and 75% specific for MACE prediction and 70% sensitive and 77% specific for ACD prediction.

Patients with plasma CT-IGFBP-4 concentrations >90 µg/L (28.3% of the group) had increased risk of future MACE [hazard ratio (HR) 2.6, P < 0.0001], and ACD (HR 6.5, P < 0.0001). CT-IGFBP-4 associated with increased risk of future ACD (HR 3.0, P = 0.004) after adjustment for the other clinical characteristics and biomarkers, including age, sex, current smoking, known hyperlipidemia, hypertension, previous known coronary artery disease, previous myocardial infarctions, heart failure, diabetes, increased CRP, Cystatin C, and PAPP-A.

Stable Coronary Disease Study. In the study 228 stable cardiovascular patients were investigated. Blood was drawn for NT- and CT-IGFBP-4 measurements after echocardiography and ergometry prior to heart catheterization. Severity and qualitative characteristics of coronary lesions vulnerability were determined angiographically.

Patient information was collected after 1108±297 days after blood sampling. Median for most of the end-points were late: 499 days for ACD and 439 for MI. Results of this study suggest that IGFBP-4 fragments are not suitable for cardiac events risk/death prediction in patients with stable cardiovascular disease. However it was clearly demonstrated that increased NT- as well as CT-IGFBP-4 are significantly associated with coronary artery disease and with the presence of B-type lesions at the moment of blood sampling.

On the basis of the results obtained from these three clinical studies circulating IGFBP-4 fragments could be used as strong predictors of middle and short-term (3 months and less) cardiac events in patients admitted to emergency department with myocardial ischemia symptoms and/or as new promising biomarkers for major adverse cardiac events and all-cause death prediction in patients with suspected acute coronary syndrome.

Patents and applications

HyTest has filed the following patent applications:

Detection of IGFBP-4 fragments as a diagnostic method

Country	Application No.
US (P)	61/221,225
FI	20095733
PCT	PCT/FI2010/050559
CN (50015)	201080029212.2
JP (50016)	2012-516812
KR (50017)	10-2011-7031706
EP (50018*)	10793670.0
US (50110)	13/381,230
HK (50995)	no number yet

References

1. **Postnikov AB, Smolyanova TI, Kharitonov AV, Serebryanaya DV, Kozlovsky SV**, et al. N-terminal and C-terminal fragments of IGFBP-4 as novel biomarkers for short-term risk assessment of major adverse cardiac events in patients presenting with ischemia. Clin. Biochem. 2012; 45(7-8): 519-24.
2. **Saenger AK, Jaffe AS**. Biomarkers of vulnerable plaque: can better ways to quantitate Pregnancy-Associated Plasma Protein A (PAPP-A) help? Clin. Biochem. 2012; 45(7-8): 517-8.

HyTest Ltd
Intelligate 1, 6th floor, Joukahaisenkatu 6
FI-20520 Turku, FINLAND

Tel. +358 2 512 0900, Fax +358 2 512 0909
E-mail: hytest@hytest.fi
www.hytest.fi