なし

2、実用新案登録
　　なし

3、その他
　　以上、特筆すべき事項なし

厚生労働科学研究費補助金（難病・がん等の疾患分野の医療の実用化研究事業）

分担研究報告書

次世代遺伝子解析による希少難治性循環器疾患の診断治療法の開発と
臨床実用化に関する研究

研究分担者　扇田久和　滋賀医科大学　生化学・分子病態生化学講座　教授

研究要旨

　遺伝性が示唆される難治性心血管系疾患症例を対象に、滋賀医科大学で採取した採血検体を用いて、大阪大学との遺伝子解析共同研究を行う。主に症例蓄積と次世代遺伝子解析シーケンサーを用いた遺伝子解析の情報解析、そしてそれによる新規希少難治性遺伝性家系の原因遺伝子同定を行う。

　次世代シーケンス解析の実践と同定遺伝子情報を診断治療に迅速に応用する独創的手法を利用し実施する。迅速なゲノム医療への応用のため、既知変異の迅速な鑑別除外により、新規変異を持つ確率の高い症例に次世代解析を行い、新規分子ないし変異に対する表現型機能解析と同定遺伝情報の臨床応用への時間短縮をはかる。難治性循環器疾患の遺伝性家系及び不整脈症例のこれまで蓄積した症例群より、適切な症例選択を行い実勢した配列解析、ゲノム情報解析結果を迅速かつ有効に診療に結実させるよう研究を進める。

A. 研究目的

　難治性不整脈疾患の原因遺伝子同定を行うことを目的とする。また、解析に際しては本研究班において開発される統一された配列解析プロトコールおよび情報解析プロトコールが、他施設に広げて行う事が可能についての比較検証が行えるようプロジェクトを進める。

　新規 rare variant（遺伝子変異）が同定された場合には、その分子機能解析と、生理的意義を知るための相互作用分子を同定し、実用化診断治療薬となる分子標的を見出すことを研究目標とする。

B. 研究方法

ゲノム解析の迅速な実施と実用的解析システムの構築

　遺伝性が示唆される難治性心血管系疾患症例（不整脈症例）を対象に、ヒトゲノム解析の説明と同意を得て、滋賀医科大学で採取した採血検体（ゲノムDNA保存）を用いて、大阪大学との共同研究を行う。

　難治性不整脈症例についてのゲノム解析をIllumina社製 GA IIx を用いて行う。データスループットは大阪大学、国立循環器病研究センターで実施している本研究のプロトコールと同一のものを用い、配列解析に関しては Hiseq2000 で行った際の解析に揃えた設定とし、情報解析は本研究用に開発したパイプライン（大阪大学 Linux サーバー）を用いてバ

リアントを検出する。

　また心筋症ゲノム解析については、大阪大学で予め Hiseq2000 を用いて配列解析を実施した検体と同一のゲノムを用いて、不整脈同様、Illumina 社製 GA IIx を用いて解析を行う。

　滋賀医科大学内で実施した心筋症および不整脈疾患の Illumina 社製 GA IIx を用いた解析結果、および心筋症ゲノム解析の滋賀医科大学と大阪大学の解析結果について、比較検討する。

（倫理）
患者情報の解析に関しては施設の倫理委員会の承認を得た上、臨床研究倫理指針を遵守し慎重におこなう。その上で患者とは個別に、医師が書面に示した計画書を明示し、十分説明をしたうえで承諾を得たもののみを本研究に使用する。特に以下の点に留意する。
①試料提供者の個人識別情報を含む情報の保護：診療情報を含めた個人情報と検体とは徹底した匿名化を行い、遺伝情報と個人情報の連結は個人識別情報管理者のみが可能となるように個人識別情報管理者をおいて情報を管理する。
②試料提供者に対する予想される危険や不利益およびそれらが生じた場合の措置：心筋生検試料採取は通常の診療の際に医学的必要に応じて行われたもののうち、診療に用いない残余検体を利用することとし、危険や不利益はないと考える。誤って遺伝情報が外部に漏洩した場合、就職・結婚・保険への加入等に関して不利益をこうむる可能性が考えられるため、これを防ぐために、個人識別情報管理者を置き、同管理者は試料の匿名化を行うとともに個人情報を厳重に管理・保管し、試料提供者のプライバシーを保護する。
③試料提供者から採取した生体材料の取り扱いについて：提供された試料は、個人識別情報管理者が連結匿名化し、匿名化ラベルのみ貼って保存する。これらの試料は、生体試料の包括利用同意を得ており、本研究だけでなく、将来倫理委員会で承認された他の自主臨床研究についても用いることが可能である。したがって検査済みの試料は、適宜連結可能匿名化番号を含む検体等を完全に削除した上で廃棄するが、使用可能な残余検体は匿名化され

たまま施錠された保管場所で保管される。また、特に研究成果として得られた情報の管理には、外部に漏洩しないように対策を行う。
動物実験においても愛護上の問題点を考慮の上、施設の審査結果を本研究について得た。この倫理規定にのっとり動物愛護上の配慮を十分行って実験をおこなう。

C. 研究結果

　難治性不整脈家系の全 Exome 解析を実施した。対象となったのは 13 検体。臨床データと連結可能匿名化されており、臨床情報をガイドに原因遺伝子の同定を行った。

　全 Exome 解析用の日本人 SNV データベースが必ずしも網羅できていない状況もあり、現時点では一つの原因遺伝子に到達することはできなかった。しかしながらシーケンスのクオリティについては、従来比較検討されてきたものと同様のデータを取ることができ、情報解析のプロトコールの実施に際しても支障なく実施することができた。

　心筋症については Hiseq2000 データおよび GA IIx の同一検体上のデータ比較も行い、一定の品質を確保できているため情報解析結果を比較することについて、不可能ではないことを確認した。

D. 考察

　独自に解析をすることと比較し、循環器ゲノム解析施設が均一な配列解析、情報解析プロトコールで解析を実施することにより、将来的にも互換性のあるデータの蓄積を行う事ができた。

　In house dataの蓄積と日本のバリアントデータベースの公開を待たなければ、小家系における原因遺伝子の同定には至らないことに変わりないが、将来の公共データベース利用が可能となった際には、同様の解析を行っている施設のデータとも参照、ないし同様の臨床家系症例を有する施設との統一解析プロトコールを用いての解析結果を蓄積していくことの意義は大きいものと考える。

また、逆に循環器施設が共同してこれら症例の蓄
積を進めていくことにより循環器疾患解析に適応さ
せたIn house genome referenceを構築することも可
能となる。これらは将来有用性の高いシステムの構
築に資するものと考えられた。

E. 結論

　難治性不整脈 13 検体について、家系症例全
Exome 解析を実施した。連結可能匿名化された臨
床データを元に、家系情報から現在利用できる手法
での原因遺伝子の絞り込みを試みた。しかし一般に
利用できる全 Exome 解析用の日本人 SNV データベ
ースはなく、現時点では一つの遺伝子に到達するこ
とはできなかった。

　In-house database および今後整備される日本人
SNV データベースの利用により原因を突き止められ
るように引き続きデータ、検体の蓄積を継続する。

　循環器ゲノム解析施設が多施設間で配列解析、
情報解析を行えるようにシステムの普及実用化を目
指す上では、均一のプロトコール上を作り、均一な
配列解析、情報解析を実施することにより、将来的
にも互換性のあるデータの蓄積を行う事が望ましい
と考えられた。

　また循環器施設が共同してこれら症例や循環器
疾患解析に適応させたIn house genome reference
の蓄積を進めていくことの重要性についても示唆的
な検討を行うことができた。

F. 健康危険情報

現在まで有害の事象なし

G. 研究発表
1、.論文発表
（英文原著）
1) An LP, Maeda T, Sakaue T, Takeuchi K,
   Yamane T, Du PG, Ohkubo I, Ogita H.
   Purification, molecular cloning and functional
characterization of swine phosphatidyl-
ethanolamine-binding protein 4 from seminal
plasma. Biochem Biophys Res Commun. 423:
690-696. 2012.

2) Ueyama H, Muraki-Oda S, Yamade S, Tanabe S,
   Yamashita T, Shichida Y, Ogita H.
   Unique haplotype in exon 3 of cone opsin
   mRNA affects splicing of its precursor, leading
   to congenital color vision defect. Biochem
   Biophys Res Commun. 424: 152-157.
   2012.

3) Hatoh T, Maeda T, Takeuchi K, Ogikubo O,
   Uchiyama S, Otsuka T, Ohkubo I, Ogita H.
   Domain 5 of high molecular weight kininogen
   inhibits collagen-mediated cancer cell
   adhesion and invasion in association with
   α-actinin-4. Biochem Biophys Res Commun.
   427: 497-502. 2012.

4) Yamane T, Hachisu R, Yuguchi M, Takeuchi K,
   Murao S, Yamamoto Y, Ogita H, Takasawa T,
   Ohkubo I, Ariga H.
   Knockdown of legumain inhibits cleavage of
   annexin A2 in the mouse kidney. Biochem
   Biophys Res Commun. 430: 482-487. 2013.

2、学会発表
1)　上山久雄、村木早苗、山出新一、田邉詔子、扇
　　田久和
　　L、M 両錐体視物質遺伝子を持つ先天色覚異
　　常－エキソン 3 の塩基多型ハプロタイプがスプ
　　ライシングのパターンに影響する　日本生化学
　　会近畿支部例会　2012 年 5 月・宇治
2)　前田利長、竹内圭介、高嶋直敬、藤吉朗、門脇
　　崇、三浦克之、上島弘嗣、扇田久和
　　ホスホリパーゼ A2 グループ 7（PLA2G7）の遺伝
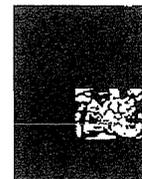　　子多型とマクロファージにおけるアポトーシス
　　誘導との相関　日本生化学会近畿支部例会
　　2012 年 5 月・宇治
3)　竹内圭介、前田利長、扇田久和
　　高分子キニノーゲン・ドメイン 5 とアクチニン-4
　　との相互作用によるがん細胞の細胞接着阻害
　　機構　日本癌学会学術総会　2012 年 9 月・札幌

4) 村木早苗、上山久雄、山出新一、田邉詔子、扇田久和、大路正人

正常遺伝子型を持つ1型2色覚の遺伝子解析
日本臨床眼科学会　2012年10月・京都

5) Takeuchi K, Majima T, Miyoshi J, Ogita H.

An Adaptor Protein Afadin Regulates Lymphatic Vessel Development by Modulating RhoA Activation. American Heart Association 2012年11月・Los Angeles, USA.

6) 上山久雄、村木早苗、豊田太、竹内圭介、扇田久和

杆体一色覚で見出された網膜錐体 cGMP 依存性カチオンチャネル α 鎖のミスセンス変異の機能的解析　日本生化学会大会　2012年12月・福岡

H. 知的財産権の出願・登録状況
（予定も含む）

1、特許取得
　　なし

2、実用新案登録
　　なし

3、その他
　　以上、特筆すべき事項なし

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

ELSEVIER

A subset of circulating microRNAs are predictive for cardiac death after discharge for acute myocardial infarction ☆

Sen Matsumoto [a,1], Yasuhiko Sakata [a,b,c,1,*], Daisaku Nakatani [a,1], Shinichiro Suna [a,1], Hiroya Mizuno [a,b,1], Masahiko Shimizu [a,1], Masaya Usami [a,1], Tatsuya Sasaki [d,1], Hiroshi Sato [e,1], Yukio Kawahara [f,1], Toshimitsu Hamasaki [g,1], Shinsuke Nanto [a,b,1], Masatsugu Hori [h,1], Issei Komuro [a,1]

[a] Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Japan
[b] Department of Advanced Cardiovascular Therapeutics, Osaka University Graduate School of Medicine, Suita, Japan
[c] Department of Evidenced-Based Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan
[d] Cardiovascular Division, Osaka Minami Medical Center, National Hospital Organization, Kawachinagano, Japan
[e] School of Human Welfare Studies Health Care Center and Clinic, Kwansei Gakuin University, Nishinomiya, Japan
[f] Laboratory of RNA Function, Osaka University Graduate School of Medicine, Suita, Japan
[g] Department of Biomedical Statistics, Osaka University Graduate School of Medicine, Suita, Japan
[h] Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan

---

ARTICLE INFO

ABSTRACT

To investigate the prognostic impact of circulating microRNAs (miRs) in patients who survived acute myocardial infarction (AMI), we compared the circulating miR signature at the time of survival discharge among samples in the serum bank of the Osaka Acute Coronary Insufficiency Study. Using a high-throughput array consisting of 667 miRs, 11 miRs were found to be differentially expressed in the serum among patients at high-risk for cardiac death. Real-time RT-PCR confirmed that the serum levels of miR-155 and miR-380* were approximately 4- and 3-fold higher, respectively, in patients who experienced cardiac death within 1 year after discharge. Accordingly, a subset of circulating miRs might be predictive for cardiac death in post-AMI patients.

© 2012 Elsevier Inc. All rights reserved.

---

1. Introduction

MicroRNAs (miRs) are small endogenous noncoding RNAs that regulate gene expression by targeting the degradation or translational repression of mRNA. Recently, it has been demonstrated that circulating miRs in the blood are useful biomarkers for cardiovascular disease [1] as well as certain forms of cancer [2]. For example, Wang et al. [3] reported that miR-208a is an excellent diagnostic marker for AMI, as demonstrated by its sensitive detection in AMI patients within 4 h of the onset of symptoms. The authors also revealed that miR-208a had high sensitivity and specificity for

diagnosing AMI by receiver operating characteristic curve analysis [4]. Kuwabara et al. [5] recently reported that circulating miR-133a serves as a useful marker for cardiomyocyte death and thus, can be used for the detection of several cardiovascular diseases, including acute myocardial infarction (AMI), and unstable angina, and takotsubo cardiomyopathy.

In patients with malignancy, the usefulness of circulating serum miRs as markers for prognosis and diagnosis has been established for several types of cancers. Few reports, however, have examined the predictive value of serum miRs in the field of cardiovascular medicine, particularly in the setting of secondary prevention after AMI. Here, we therefore investigated whether circulating miRs collected during the convalescent stage of AMI could predict cardiac death in post-AMI patients registered in the Osaka Acute Coronary Insufficiency Study (OACIS) database.

2. Materials and methods

2.1. OACIS registry

The OACIS is a prospective, multicenter observational study enrolling consecutive AMI patients in 25 collaborating hospitals from the Osaka region of Japan, and is registered with the

---

University Hospital Medical Information Network Clinical Trials Registry, Japan (ID: UMIN000004575). A detailed description of the OACIS has been published elsewhere [6]. The present study protocol was approved by the ethics committee of each participating hospital.

2.2. Patients

Among 8603 patients with AMI who were registered in the OA-CIS between 1998 and 2009, we firstly selected 4160 consecutive patients fulfilling the following criteria: (1) discharged alive and (2) provided written informed consent for serum analysis at the time of registration. Among the selected patients, 60 cardiac deaths occurred after discharge. In the discovery phase, we randomly selected 7 patients who died of cardiac cause within a year after discharge and another 7 patients who did not experience any cardiovascular events during a 3-year follow-up period using propensity score-based matching of age, gender, classical coronary risk factors, infarction size, reperfusion therapy, and medical treatment at discharge. In the validation phase, we increased the number of patients in the cardiac death and survival groups to 19 and 21, respectively.

2.3. Serum collection

At each hospital, fasting blood samples were collected into serum separator tubes, which were then centrifuged at 1430g for 15 min at 4 °C to separate the clots. Serum was removed from the tubes and stored at −80 °C until the time of the assay.

2.4. RNA isolation and miR analysis

Total RNA was isolated from 1 ml of serum using a mirVana Paris kit (Life Technologies Co., Carlsbad, CA). Reverse transcription and preamplification steps were performed with a TaqMan MicroRNA RT kit (Life Technologies Co.) and Megaplex Primers (Life Technologies Co.). To identify miRs that could serve as predictive markers of cardiac death at 1 year, the expression levels of 667 miRs were compared between groups using TaqMan Human MicroRNA A and B Arrays, version 2.0 (Life Technologies Co.) (discovery phase). To confirm the results from the discovery phase, the expression levels of candidate miRs were examined by real-time PCR using a 7900HT Fast Real-Time PCR system (validation phase).

2.5. Data collection

Research cardiologists and trained research nurses or coordinators recorded data concerning sociodemographic variables, medical history, therapeutic procedures, and clinical events during patients' hospital stays. Clinical data after discharge were obtained at 3 and 12 months after the onset of AMI, and annually thereafter. The incidence of cardiac death was the clinical endpoint of the study.

2.6. Statistical analysis

To adjust for potential confounding factors, we selected two groups for the discovery and validation phases using a propensity score-based method. Briefly, a propensity score for cardiac death within 1 year after discharge was calculated using logistic regression analysis that included age, gender, diabetes mellitus (DM), hypertension (HT), dyslipidemia, smoking, previous MI, Killip class ≥ II at admission, infarction size, reperfusion therapy, and medication at discharge (ACEI or ARB, and statin) as variables. For the analysis, we first selected seven patients who died of cardiac cause within 1 year after discharge and another seven patients

who did not experience any cardiovascular events during a 3-year follow-up period. We then selected 19 patients who died of cardiac cause after discharge and 21 patients who did not experience any cardiovascular events during a 2-year follow-up period. For the two sets of groups, patient backgrounds were compared using the χ^2 test. Expression levels of miRs between the two groups were analyzed by the Mann–Whitney U test. Associations were considered significant if the p value was <0.05. All statistical analyses were performed using SPSS software (SPSS Japan, Inc., Tokyo, Japan).
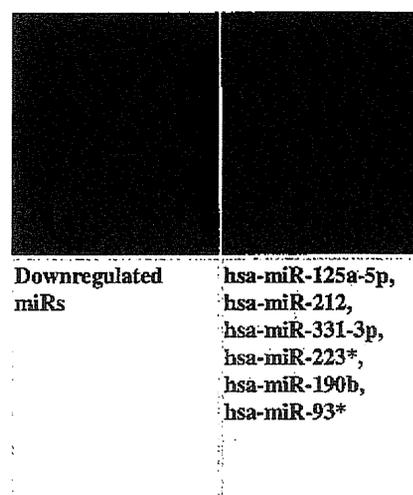
3. Results

3.1. Discovery phase

To investigate whether serum miRs could predict prognosis in the convalescent stage of AMI, we compared circulating miR signatures at the time of survival discharge using the OACIS serum bank. As shown in Table 1, patient backgrounds were well matched between patients who died of cardiac cause within 1 year after discharge ($N = 7$) and those who did not experience any cardiovascular events during the 3-year follow-up period ($N = 7$) in the discovery phase. High-throughput array analysis revealed

Table 1
Baseline characteristics in the discovery phase.

Variable	Cardiac death ($N = 7$)	Event free ($N = 7$)	p Value
Age (years)	68 ± 8	67 ± 7	0.810
Men (%)	86	71	1.000
Diabetes mellitus (%)	57	29	0.592
Hypertension (%)	83	57	0.559
Dyslipidemia (%)	71	57	1.000
Smoking (%)	57	86	0.559
Previous MI (%)	14	0	1.000
Peak CK ≥ 3000 IU/L (%)	14	43	0.559
Killip class ≥ II on admission (%)	43	43	1.000
Reperfusion therapy (%)	100	100	–
ACEI or ARB (%)	71	67	1.000
Statin (%)	57	67	1.000

ACEI: angiotensin-converting enzyme inhibitor, ARB: angiotensin receptor blocker, CPK: creatinine phosphokinase, MI: myocardial infarction.

Downregulated miRs	hsa-miR-125a-5p, hsa-miR-212, hsa-miR-331-3p, hsa-miR-223*, hsa-miR-190b, hsa-miR-93*

Fig. 1. High-throughput array analysis revealed that the levels of 5 miRs were increased and those of 6 miRs were decreased in the cardiac death group.

Table 2
Baseline characteristics in the validation phase.

Variable	Cardiac death (N = 19)	Event free (N = 21)	p Value
Age (years)	72 ± 12	69 ± 10	0.467
Men (%)	74	76	1.000
Body mass index (kg/m²)	24.4 ± 3.51	23.0 ± 3.50	0.227
Diabetes mellitus (%)	53	62	0.750
Hypertension (%)	83	76	0.702
Dyslipidemia (%)	47	48	1.000
Smoking (%)	53	71	0.328
Previous MI (%)	21	20	1.000
Onset to admission time <24 h (%)	72	75	1.000
Peak CK≥3000 IU/L (%)	42	44	1.000
Killip class ≥II on admission (%)	41	29	0.502
Reperfusion therapy (%)	95	100	0.475
Multivessel disease (%)	63	52	0.538
ACEI or ARB (%)	74	60	0.501
Beta blocker (%)	68	60	0.741
Statin (%)	42	60	0.343
Antiplatelet therapy (%)	95	100	0.487

ACEI: angiotensin-converting enzyme inhibitor, ARB: angiotensin receptor blocker, CPK: creatinine phosphokinase, MI: myocardial infarction.

that 11 miRs were differently expressed between the two patient groups. The identified miRs were selected as initial candidates for the validation study (Fig. 1).

3.2. Validation phase

In the validation phase (cardiac death group, N = 19; and survival group, N = 21), real-time RT-PCR confirmed that 2 out of 11 miRs identified in the discovery phase were increased in the cardiac death group (N = 19) as compared with the survival group (N = 19). The serum levels of miR-155 and miR-380* were approximately 4- and 3-fold higher, respectively, in the cardiac death group, whereas the serum levels of the other 9 miRs differentially expressed in the discovery phase analysis were comparable between the two groups (Table 2, Fig. 2).

4. Discussion

To our knowledge, this is the first study to investigate whether circulating miRs are associated with prognosis in the field of cardiovascular medicine. Specifically, we examined the association between serum levels of 667 miRs and future cardiac events in post-AMI patients and found that serum levels of miR-155 and miR-380* in the convalescent stage of AMI were higher in patients who subsequently experienced cardiac death in 1 year. Although further investigation is required to confirm the predictive value of these miRs, our findings suggest the intriguing possibility that circulating miRs can serve as prognostic biomarkers for cardiovascular diseases.

MiRs are small endogenous RNAs that play important roles in animals and plants by targeting mRNAs for degradation or translational repression [7]. Dysregulation and tissue-specific patterns of intracellular miR expression have been reported in various diseases, particularly for several types of cancers [8]. In addition, miRs appear to circulate in the blood in a relatively stable form [9], suggesting that miRs may have biological functions outside the cell and thus, can potentially serve as diagnostic or prognostic biomarkers for cancer, as well as therapeutic targets. With regard to cardiovascular diseases, however, the potential of miRs as diagnostic markers has only recently been proposed [1], and few prognostic features or therapeutic potentials of circulating miRs have been reported.

In the present study, we found that the serum levels of miR-155 and miR-380* at the time of discharge after AMI were approxi-

mately 4- and 3-fold higher in patients who subsequently died of cardiac cause within 1 year of discharge than those in patients who did not experience cardiovascular events during the 3-year follow-up period.

This observation is of clinical significance, because it indicates serum miRs have the potential to predict prognosis in patients with cardiovascular disease, and also suggests that these miRs have the potential to be directly involved in future treatment approaches. Unlike studies investigating patients with malignancy [10], previous studies failed to identify miRs detected in ACS patients as therapeutic targets, possibly because such miRs were likely released into the circulation as a result of myocardial necrosis.

Although the underlying mechanism for the association between elevated serum levels of miR-155 and the increased risk for cardiac death after survival discharge of AMI is unclear, several explanations are possible. For example, Martin et al. [11] recently demonstrated that miR-155 directly interacts with the 3'-untranslated region of angiotensin II type 1 receptor (AT1R) mRNA, thereby modulating expression of AT1R and angiotensin II-induced extracellular signal-related kinase 1/2 (ERK1/2) activation. In addition, the expression levels of miR-155 are increased by angiotensin II in atherosclerotic cells in vitro (data not shown). This finding suggests that serum miR-155 levels may be increased through activation of the renin angiotensin system and thus, be associated with prognosis in post-AMI patients. Another possibility for elevated miR-155 in serum is as a result of inflammation. Yao et al. [12] reported that miR-155 is processed from BIC, a non-coding transcript that is highly expressed in both activated B and T cells, and monocytes/macrophages. Therefore, serum miR-155 levels might be increased following activation of monocytes/macrophages, which could lead to cardiovascular events. Similarly, elevated levels of serum miR-380* might reflect activation of p53 in failed myocardium, because miR-380-5p is reported to repress p53 expression via a conserved sequence in the p53 3'-untranslated region [13]. As up-regulation of the p53 pathway is one of the major causes for the development of heart failure in mouse models of pressure-overload and AMI [14], miR-380* might be secreted into the circulation from p53 up-regulated myocardium as a negative feedback loop of the p53 pathway, and thus be associated with prognosis after AMI.

Several limitations of this study warrant mention. First, this was a retrospective analysis using a small sample size of AMI patients selected from a prospective observational study. Second, our
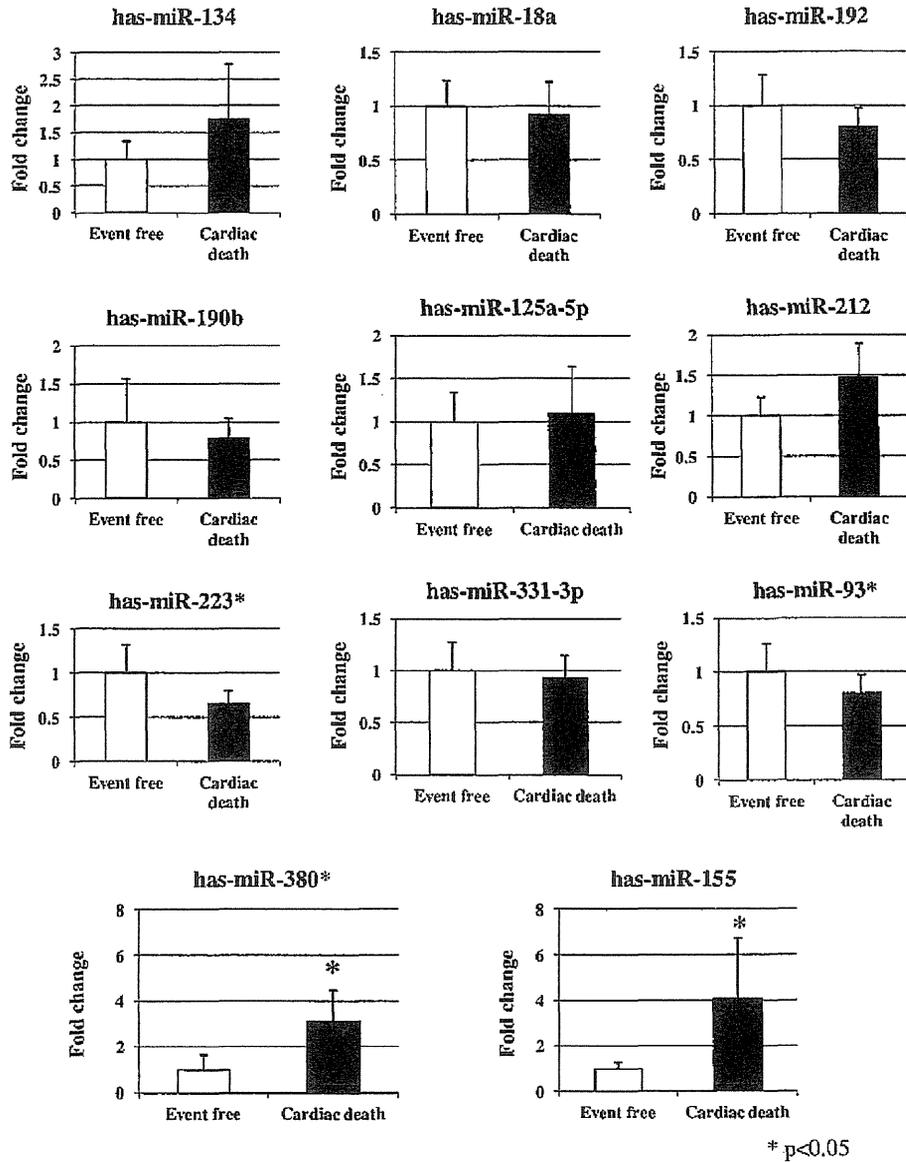
Fig. 2. Serum levels of miR-155 and miR-380* were approximately 4- and 3-fold higher, respectively, in the cardiac death group, whereas the serum levels of the 9 other examined miRs were between the two groups.

analysis was unable to detect a direct cause-effect relationship between the elevation of serum miR levels and cardiac death in post-AMI patients. Due to these limitations, further studies are warranted to confirm the present results.

Acknowledgments

Appendix A

A.1. The OACIS Head Office

Chair: Issei Komuro, Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita 565-0871, Japan.

Secretariats: Yasuhiko Sakata (Chief), Daisaku Nakatani, Shinichiro Suna, Masaya Usami, Sen Matsumoto, Masahiko Hara, Mariko Kishida, Rie Nagai: Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Japan.

Statisticians: Toshimitsu Hamasaki, Kenichi Hayashi, Toshifumi Sugitani: Department of Biomedical Statistics, Osaka University Graduate School of Medicine, Suita, Japan.

A.2. The OACIS Investigators (listed in alphabetical order for institutions)

Yoshiyuki Kijima, Yusuke Nakagawa, Minoru Ichikawa, Higashi-Osaka City General Hospital, Higashi-Osaka, Japan; Young-Jae Lim, Shigeo Kawano, Hideyuki Nanmori, Kawachi General Hospital, Higashi-Osaka, Japan; Hiroshi Sato, Kwasnsei Gakuin University, Nishinomiya, Japan; Takashi Shimazu, Hisakazu Fuji, Kazuhiro Aoki, Kobe Ekisaikai Hospital, Kobe, Japan; Masaaki Uematsu, Yoshio Ishida, Tetsuya Watanabe, Masashi Fujita, Masaki Awata, Kansai Rosai Hospital, Amagasaki, Japan; Michio Sugii, Meiwa Hospital, Nishinomiya, Japan; Masatake Fukunami, Takahisa Yamada, Takashi Morita, Osaka General Medical Center, Osaka, Japan; Shinji Hasegawa, Nobuyuki Ogasawara, Osaka Kosei Nenkin Hospital, Osaka, Japan; Tatsuya Sasaki, Yoshinori Yasuoka, Kiyoshi Kume, Osaka Minami Medical Center, National Hospital Organization, Kawachinagano, Japan; Hideo Kusuoka, Yukihiro Koretsune, Yoshio Yasumura, Keiji Hirooka, Osaka Medical Center, National Hospital Organization, Osaka, Japan; Masatsugu Hori (previous Chair), Osaka Prefectural Hospital Organization Osaka Medical Center for Cancer and Cardiovascular Diseases; Kazuhisa Kodama, Yasunori Ueda, Kazunori Kashiwase, Mayu Nishio, Osaka Police Hospital, Osaka, Japan; Yoshio Yamada, Jun Tanouchi, Masami Nishino, Hiroyasu Kato, Ryu Shutta, Osaka Rosai Hospital, Sakai, Japan; Shintaro Beppu, Hiroyoshi Yamamoto, Osaka Seamens Insurance Hospital, Osaka, Japan; Issei Komuro, Shinsuke Nanto, Yasushi Matsumura, Satoru Sumitsuji, Yasuhiko Sakata, Shungo Hikoso, Daisaku Nakatani, Masahiro Kumada, Michihiro Takeda, Shinichiro Suna, Osaka University Graduate School of Medicine, Suita, Japan; Toru Hayashi, Yasuji Doi, Ken-ichiro Okada, Noritoshi Ito, Saiseikai Senri Hospital, Suita, Japan; Kenshi Fujii, Katsuomi Iwakura, Atsushi Okamura, Motoo Date, Yoshiharu Higuchi, Koji Inoue, Sakurabashi Watanabe Hospital, Osaka, Japan; Noriyuki Akehi, Settsu Iseikai Hospital, Settsu, Japan; Eiji Hishida,Teramoto Memorial Hospital, Kawachinagano, Japan; and Shiro Hoshida, Kazuhiko Hashimura, Takayoshi Adachi, Yukinori Shinoda, Yao Municipal Hospital, Yao, Japan.

References

[1] Y. D'Alessandra, P. Devanna, F. Limana, et al., Circulating microRNAs are new and sensitive biomarkers of myocardial infarction, Eur. Heart. J. 31 (2010) 2765–2773.
[2] C.H. Lawrie, S. Gal, H.M. Dunlop, et al., Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma, Br. J. Haematol. 141 (2008) 672–675.
[3] G.K. Wang, J.Q. Zhu, J.T. Zhang, et al., Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans, Eur. Heart. J. 31 (2010) 659–666.
[4] C.E. Metz, Basic principles of ROC analysis, Semin. Nucl. Med. 8 (1978) 283–298.
[5] Y. Kuwabara, K. Ono, T. Horie, et al., Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage, Circ. Cardiovasc. Genet. 4 (2011) 446–454.
[6] Y. Sakata, D. Nakatani, M. Shimizu, et al., Oral treatment with nicorandil at discharge is associated with reduced mortality after acute myocardial infarction, J. Cardiol. 59 (2012) 14–21.
[7] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[8] T. Ueda, S. Volinia, H. Okumura, et al., Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis, Lancet. Oncol. 11 (2010) 136–146.
[9] P.S. Mitchell, R.K. Parkin, E.M. Kroh, et al., Circulating microRNAs as stable blood-based markers for cancer detection, Proc. Nat. Acad. Sci. USA 105 (2008) 10513–10518.
[10] C. Liu, K. Kelnar, B. Liu, et al., The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44, Nat. Med. 17 (2011) 211–215.
[11] M.M. Martin, E.J. Lee, J.A. Buckenberger, et al., MicroRNA-155 regulates human angiotensin II type 1 receptor expression in fibroblasts, J. Biol. Chem. 281 (2006) 18277–18284.
[12] R. Yao, Y. Ma, Y. Du, et al., The altered expression of inflammation-related microRNAs with microRNA-155 expression correlates with Th17 differentiation in patients with acute coronary syndrome, Cell. Mol. Immunol. 8 (2011) 486–495.
[13] A. Swarbrick, S.L. Woods, A. Shaw, et al., MiR-380-5p represses p53 to control cellular survival and is associated with poor outcome in MYCN-amplified neuroblastoma, Nat. Med. 16 (2010) 1134–1140.
[14] M. Sano, T. Minamino, H. Toko, et al., P53-induced inhibition of Hif-1 causes cardiac dysfunction during pressure overload, Nature 446 (2007) 444–448.

Design and Rationale of Low-Dose Erythropoietin in Patients with ST-Segment Elevation Myocardial Infarction (EPO-AMI-II Study): A Randomized Controlled Clinical Trial

Tetsuo Minamino · Ken Toba · Shuichiro Higo ·
Daisaku Nakatani · Shungo Hikoso · Masao Umegaki ·
Kouji Yamamoto · Yoshiki Sawa · Yoshifusa Aizawa ·
Issei Komuro · EPO-AMI-II study investigators

Abstract

Purpose The development of novel pharmaceutical interventions to improve the clinical outcomes of patients with acute ST-segment elevation myocardial infarction (STEMI) is an unmet medical need worldwide. In animal models, a single intravenous administration of erythropoietin (EPO) during reperfusion improves left ventricular (LV) function in the chronic stage. However, the results of recent proof-of-concept trials using high-dose EPO in patients with STEMI are inconsistent. In our pilot study, low-dose EPO after successful percutaneous coronary intervention (PCI) improved the LV ejection fraction (EF) and did not trigger severe adverse clinical events in patients with STEMI. One possible reason for this discrepancy is the dose of EPO used.

Methods and results We have started a double-blind, placebo-controlled, randomized, multicenter clinical trial (EPO-AMI-II) to clarify the safety and efficacy of low-dose EPO in patients with STEMI. STEMI patients who have a low LVEF (<50 %) will be randomly assigned to intravenous administration of placebo or EPO (6,000 or 12,000 IU) within 6 h after successful PCI. The primary endpoint is the difference in LVEF between the acute and chronic phases (6 months), as measured by single-photon emission computed tomography. The patient number needed for EPO-AMI-II is 600. The study will stop when superior efficacy or futility is detected by an interim analysis. This study has been approved by the Evaluation System of Investigational Medical Care.

Conclusions EPO-AMI-II study will clarify the safety and efficacy of low-dose EPO in STEMI patients with LV dysfunction in a double-blind, placebo-controlled, multicenter study. (247 words)

Key words Erythropoietin · Low-dose · Acute myocardial infarction · LV dysfunction

T. Minamino · S. Higo · D. Nakatani · S. Hikoso · Y. Sawa · I. Komuro (✉)
Department of Cardiovascular Medicine,
Osaka University Graduate School of Medicine,
2-2 Yamada-oka,
Suita, Osaka 565-0871, Japan
e-mail: komuro-tky@umin.ac.jp

K. Yamamoto
Department of Biomedical Statistics,
Osaka University Hospital,
Suita, Japan

M. Umegaki
Medical Center for Translational Research, Osaka University Hospital,
Suita, Japan

K. Toba
First Department of Internal Medicine,
Niigata University Medical and Dental Hospital,
Niigata, Japan

Y. Aizawa
Tachikawa Medical Center,
Nagaoka, Japan

Despite improved clinical outcomes by early reperfusion with thrombolysis and primary percutaneous coronary intervention (PCI) with stenting, the mortality of patients with

ST-segment elevation myocardial infarction (STEMI) is still high in Western countries and Japan [1, 2]. Furthermore, in the chronic stage after MI, heart failure can develop due to left ventricular (LV) remodeling [3]. To date, most clinically tested agents that induce cardioprotection have failed to reduce infarct size in clinical settings [4]. Thus, novel pharmaceutical interventions to improve the clinical outcomes of patients with STEMI are urgently needed. Animal studies show that the intravenous administration of erythropoietin (EPO), a glycoprotein hormone consisting of 165 amino acid residues [5], at the onset of reperfusion reduces the myocardial infarct size and prevents cardiac remodeling, with enhanced neovascularization in the heart after MI [6, 7]. Several proof-of-concept studies have been performed to clarify the cardioprotective effects of EPO in patients with STEMI. The administration of high-dose EPO (60,000–99,000 IU) did not improve left ventricular ejection fraction (LVEF) or reduce infarct size [8–10]. Regarding secondary endpoints, the use of EPO has been associated with a trend toward an increase in major adverse cardiovascular events in 2 studies [8, 10] and significantly fewer events in a third study [9]. In contrast, low-dose EPO is likely to be cardioprotective, according to small clinical trials [11–13]. Platelet activation by high-dose EPO [14] and the existence of an optimal dose for limiting infarct size [15] may explain the dose-dependent discrepancy of EPO-induced cardioprotection. Importantly, pilot studies showed that low-dose EPO is associated with improved left ventricular function without major adverse cardiovascular events [11, 12]. Furthermore, our post-hoc analysis revealed that EPO administration was highly associated with improved LV function in STEMI patients with a low LV ejection fraction (LVEF) (<50 %) (Fig. 1).

Therefore, we have started a double-blind, placebo-controlled, randomized, multicenter clinical trial (EPO-AMI-II) to clarify the safety and efficacy of low-dose EPO in STEMI patients with a low LVEF (<50 %). The protocol was submitted to the Evaluation System of Investigational Medical Care of the Ministry of Health, Labour and Welfare of Japan and was approved under the Japanese governmental health insurance system on 1 August 2011.

Methods

Study objects

The objectives of this study are to evaluate whether a single bolus administration of EPO prevents ischemia-reperfusion injury dose-dependently and to estimate the optimum clinical dose of EPO in patients with STEMI after successful PCI by analyzing the improvement in LVEF between the acute and chronic stages.

Study design

EPO-AMI-II is an ongoing multicenter, prospective, randomized, double-blind, placebo-controlled, dose-finding study in patients presenting with a first STEMI. After a successful PCI, patients will be randomly assigned to receive either an intravenous bolus dose of epoetin-beta (EPO) (6,000 or 12,000 IU) or placebo on top of standard medical care (Fig. 2). This trial was registered at the UMIN Clinical Trials Registry as UMIN000005721.
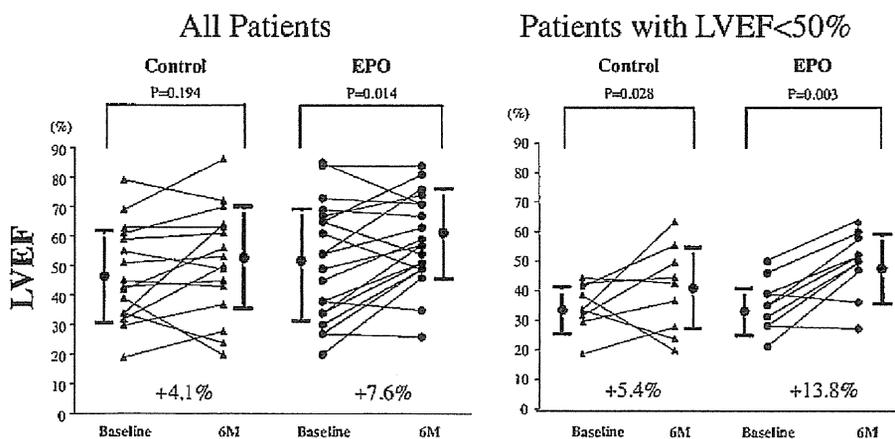
Fig. 1 Post-hoc analysis of the EPO-AMI-I results. Panel a shows the LVEF between the acute and chronic stages in all patients in the EPO-AMI-I study. EPO, but not saline, administration significantly increased LVEF at 6 months after an MI. Panel b shows the LVEF between the acute and chronic stages in patients with LVEF <50 % in the EPO-AMI-I study. Both saline and EPO significantly increased LVEF at 6 months after an MI. The improvement of LVEF did not significantly differ between the saline- and EPO-treated groups. See the abbreviation definitions in the text
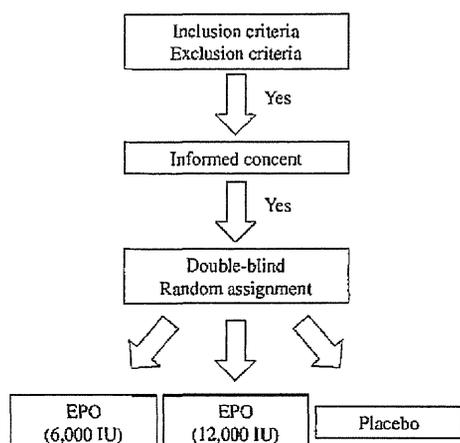
Fig. 2 Study flow chart

Table 1 Inclusion and exclusion criteria

Inclusion criteria

1. Patients with first-time myocardial infarction

2. Patients with ST-elevation acute myocardial infarction (AMI) who have successful reperfusion by PCI within 12 h after the symptom onset

3. Patients whose ejection fraction at enrollment is <50 % on UCG or LVG

4. Age: over 20 years old, under 80 years old

5. Patients who agreed with participation to the trial in writing

Exclusion criteria

1. Patients with significant stenotic lesions in non infarct-related artery which require revascularization

2. Patients who resulted in obviously impaired reperfusion

3. Patients with Killip class III or IV, or cardiogenic shock at admission

4. Patients with advanced renal or hepatic dysfunction (Cre more than 2 mg/dl, or T-Bil more than 3 mg/dl)

5. Patients with blood pressure more than 140/90 mmHg after PCI

6. Hematocrit more than 54 % on admission

7. Patients who exhibit atrial fibrillation after PCI

8. Patients who have been diagnosed with malignant hypertension

9. Patients who have previously received treatment with EPO

10. Patients who received a blood transfusion in the last 3 months

11. Patients who are or have been diagnosed with cancer in the past 5 years

12. Patients who are complicated with severe infection such as pneumonia or sepsis

13. Patients who are contraindicated to aspirin or thienopyridine derivatives

14. Women who are pregnant, breastfeeding, or have a possibility for pregnancy

15. Patients whom researchers judged that they are not appropriate to participate this trial

Patients

Consecutive patients with diagnostic signs and symptoms of an acute MI who satisfy the study inclusion and exclusion criteria (Table 1). After successful PCI, patients will be asked for written informed consent, and if they agree, will be assigned according to a pre-defined central web-based randomization system to receive EPO or placebo on top of optimal standard medical care. Patients will receive the study drug within 6 h after PCI. The patient, the attending physician, and the staff performing SPECT and the clinical follow-up will be unaware of the assigned treatment.

End points

The primary end point of this study is to evaluate the LVEF improvement between the acute (days 4–7) and chronic stages (6 months) (Table 2). The secondary end points of this study are to evaluate the efficacy and safety of EPO treatment. The efficacy is evaluated by analyzing indices of cardiac function 6 months after EPO administration. These are calculated with electrocardiogram-gated single-photon emission computed tomography (SPECT) and include LV end-diastolic volume (LVEDV), LV end-systolic volume (LVESD), LVEDV index, LVESV index, regional wall motion score, % uptake at resting, and defect size. The survival ratio, cardiovascular events (defined as cardiac death, stroke, nonlethal myocardial infarction, admission due to worsening of heart failure or unstable angina, revascularization, and onset of heart failure symptoms), and NT-ProBNP at the 6-month follow-up will also be analyzed to evaluate the efficacy of EPO treatment (Table 2). The safety is based on the incidence of major adverse events, clinical laboratory test data and vital signs.

Study drug administration

Prior to or at the time of primary PCI, standard antithrombotic treatments for acute MI are administered. Within 6 h after PCI, the enrolled patients are randomly assigned to placebo or an Epo dose (6,000 or 12,000 IU). Active drug or placebo is diluted in 10 mL of saline and administered intravenously over 1 min. The double-blind administration is ensured by a subject identification code unknown to physicians, nurses and patients. Drug or placebo is prepared under medical supervision according to instructions contained in predefined packages provided by the EPO-AMI-II organization. Standard treatment, including beta-blockade, lipid-lowering therapy, and angiotensin-converting enzyme inhibition or angiotensin-II receptor blockade, is additionally prescribed. EPO and placebo are kind gifts of Chugai Pharmaceutical Co. Ltd (Tokyo, Japan).

Table 2 Primary and secondary end points

Primary end point
The improvement of left ventricular ejection fraction at the chronic phase (the mean of differences between LVEF value at 4–7 days and that at 6 months after administration)
Secondary end point
[Efficacy]
1. Indexes of cardiac function 6 months after administration of epoetin-beta, which are calculated with cardiac scintigraphy (LVEDV, LVESV, LVEDVI, LVESVI, regional wall motion score, ischemia and defect size (SRS (Summed rest Score), SDS (Summed difference Score), %Defect Size, %uptake at resting))
2. Survival ratio
3. Cardiac event ratio (Cardiac death, stroke, nonlethal myocardial infarction, admission due to worsening of heart failure or unstable angina, revasculization, onset of heart failure symptoms (typical dyspnea at rest or during exercise, pulmonary congestion or pretibial edema)
4. NT-ProBNP 6 months after administration
[safety]
1. Adverse events
2. Laboratory test data
3. Vital signs (blood pressure, pulse rate)

Clinical and laboratory measures

Blood pressure, heart rate, and ECG are monitored at regular intervals until discharge (Fig. 3). Major adverse events (as defined above) are recorded during hospitalization and up to 6 months thereafter. At 4–7 days after admission and at 6 months, cardiac SPECT is also performed to evaluate cardiac function.

Quantification of LV function and infarct size

We will perform ECG-gated 99mTc-MIBI SPECT 4–7 days after PCI as the baseline measurement and at the 6-month follow-up. The 99mTc-MIBI (600–740 MBq) is administered at baseline and at the 6-month follow-up. SPECT image acquisition is performed 60 min after the 99mTc-MIBI injection. ECG-gated SPECT is performed after the administration of 99mTc-MIBI at rest. In ECG gating, SPECT data divided into 16 equal intervals are analyzed using Quantitative Gated SPECT software (Cedars-Sinai Medical Center, Los Angeles, CA, USA), which is also used

to calculate EDVI, ESVI and LVEF. Pharmacologic stress tests are performed with non-gated 99mTc-MIBI SPECT. Adenosine (Adenoscan; DAIICHI SANKYO, Tokyo, Japan) is administered at a rate of 0.72 mg/kg for 6 min. The 99mTc-MIBI is injected 3 min after the start of adenosine infusion. The non-gated SPECT image is used to assess the severity of myocardial perfusion abnormalities, and regional uptake and the infarct area are calculated using Quantitative Perfusion SPECT software (Cedars-Sinai Medical Center). Regional uptake is assessed by applying a 17-segment model of the left ventricle according to the standard myocardial segmentation of the Cardiac Imaging Committee of the Council on Clinical Cardiology of the American Heart Association. Regional uptake is expressed as the mean uptake count in these segments. Defects at less than the threshold of 60 % of peak counts are identified as infarcted myocardium, and the infarct area is expressed as a percentage of the entire left ventricle involved. SPECT data will be analyzed in a blinded fashion by the SPECT Core Center members with the assistant of nuclear medicine special radiological technologist at MICRON Co., Ltd (Molecular Imaging CRO Network, Tokyo, Japan.). Finally, the analyzed data will be evaluated by an independent RI assessment committee.

Adverse events and additional safety assessments

An independent data safety monitoring board (DSMB) will receive real-time clinical information and will perform interim safety and efficacy analyses at 33 %, 66 % and 100 % recruitment. There are no formal (statistical) rules for stopping treatment due to safety reasons in this study. The DSMB recommendations are based on a clinical assessment of the frequency, and the nature of the serious adverse events and their relation to the investigational treatment.

Sample size calculation

Based on the results of our pilot study in STEMI patients with LVEF <50 % (LVEF improvement in the EPO-II group: 13.80 ± 9.85 %, $n=11$, and in the placebo group: 5.44 ± 14.80 %, $n=9$) (Fig. 1), the difference in LVEF improvement between the EPO (12,000 IU) treatment group and the placebo group is estimated to be 4.42 % with a common standard deviation of 14.33 %. As a result, the effect size is estimated to be 0.31 [16]. To demonstrate the

Fig. 3 Study schedule
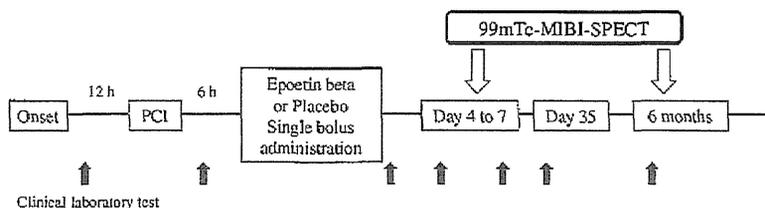
treatment difference with a power of 0.85 and a 1-sided alpha of 0.025, 190 patients per group will be needed. However, because we plan to perform two interim analyses, we will need 193 patients per group [17]. Taking into account several patients dropping out, the total sample size to be recruited will be 200 patients in each treatment group, i.e., 600 patients will be recruited in this study.

Interim analysis

There will be two formal interim analyses on the safety and efficacy of the primary end point: after 198 and 396 randomized patients are enrolled and followed up for 6 months. For the interim analyses on efficacy, the DSMB will evaluate the primary end point using the Lan-DeMets method with the O'Brien-Fleming spending function. Asymmetric stopping boundaries are planned, with early termination of the study recommended in the event of evidence of overwhelming benefit (2-sided $P<.001$ favoring EPO) or substantive harm (2-sided $P<.01$ against EPO) once sufficient events have accrued.

Statistical analysis

Data will be analyzed based on an intention-to-treat principle. The efficacy end point is LVEF improvement. The null hypothesis, that all treatment groups will have the same mean LVEF improvement, will be tested against the alternative hypothesis, that the mean LVEF improvement in the treatment groups will increase in the order of placebo, EPO (6,000 IU) and EPO (12,000 IU), according to the contrast test with a contrast coefficient (−1, 0, 1) based on the t-statistic. The contrast test will be evaluated based on a 1-sided significance level of 0.025. The secondary efficacy end point of OS in each group will be analyzed by the Kaplan-Meier method and compared using the log-rank test. Cardiovascular events and NT-ProBNP at the 6-months follow-up will be analyzed by a nonparametric test (e.g., Wilcoxon rank sum test). Safety analyses will be performed to summarize the adverse events in each treatment group. The baseline characteristics of the study patients will be summarized using frequencies and percentages for categorical variables and using means with standard deviations for continuous variables.

Current status

EPO-AMI-II began enrolling patients in December 2011. As of May 15, 2012, the application for the Evaluation System of Investigational Medical Care is ongoing, and 14 of 24 eligible centers have been approved. Completion of study enrollment is targeted for September 30, 2014.

Allowing for the 6-month follow-up of the final randomized patient, trial completion is anticipated by March 2015.

Discussion

We have started the EPO-AMI-II study to clarify the safety and efficacy of low-dose EPO in the improvement of LVEF in STEMI patients with a low LVEF (<50 %). EPO-AMI-II is a multicenter, prospective, randomized, double-blind, placebo-controlled, dose-finding study in patients with their first STEMI.

Randomized clinical studies to clarify the effects of low-dose EPO in patients with STEMI

Therapies that can reduce myocardial damage and augment neovascularization in the heart after an MI may be beneficial in patients with STEMI. Experimental studies demonstrate that the intravenous administration of EPO at the onset of reperfusion reduces myocardial infarct size and prevents cardiac reverse remodeling, with enhanced neovascularization in the heart after an MI [6, 7]. Recently, proof-of-concept studies using high-dose EPO have reported inconsistent cardioprotection results from EPO in patients with STEMI (Table 3). The use of high-dose EPO at the time of reperfusion for an acute MI to salvage the myocardium or to improve LV function will not be further pursued in any newly initiated study.

In contrast, low-dose EPO is likely to be cardioprotective in small clinical trials [11–13]. Potential mechanisms to explain the dose-dependent discrepancy of EPO in cardioprotection may be attributable to platelet activation and the existence of an optimal dose for limiting infarct size. Platelet activation by a high dose of EPO [14] and the existence of an optimal dose for limiting infarct size [15] may explain the dose-dependent discrepancy of EPO-induced cardioprotection. Because EPO has structural similarity with thrombopoietin, high-dose EPO increases platelet production and reactivity, which leads to an increased risk of thrombosis and cardiovascular events. Additionally, a dose response curve of the bioactivity of cytokines does not necessarily appear to be guided by a sigmoid function. Positive intracellular signal of cytokine receptors via serial chain reaction of protein tyrosine kinases is typically interfered by automated circuit reaction of protein tyrosine phosphatase such as SHP1 to avoid overdoing of growth and inflammation [18]. In fact, administration of high-dose EPO lost its cardioprotective activity in rat and mouse coronary ischemia/reperfusion models [15, 19]. The rationale for EPO treatment for

Table 3 Overview of randomized controlled studies investigating the effects of EPO in patients with acute myocardial infarction

Trial	Dose of EPO	Primary outcome	Result	Cardiovascular event
REVIVAL-3	33,333 IU×3 (0, 24, 48 h)	LV EF	No change	Increase (not significant)
HEBE-III	60,000 IU	Infarction size	No change	Decrease (significant)
REVEAL	60,000 IU	Infarction size	No change	Increase (not significant)
EPOC-AMI	6,000 IU×3 (day 0, 2, 4)	LV EF	Improve	No change
EPO-AMI-I	12,000 IU	LV EF	Improve	No change
EPO-AMI-II	6,000 or 12,000 IU	LV EF		

patients with STEMI lies in the low-dose EPO trials, although these have only been small clinical trials to date.

Protocol of EPO-AMI-II study

On the basis of a post-hoc analysis of our pilot study (EPO-AMI-I) and a recent proposal from workshops [20–22], we have modified the protocol for the EPO-AMI-II study. First, we created new inclusion criteria to include patients with an LVEF <50 %. Only patients who have large myocardial infarcts can receive benefits from any adjunctive therapy [23, 24]. Consistently, the post-hoc analysis of the EPO-AMI-I study revealed that STEMI patients with an LVEF <50 % received large benefits from EPO administration11 (Fig. 1). When patients with significant stenotic lesions in non-infarct-related arteries that required revascularization were excluded, more than 90 % of STEMI patients who met the inclusion and exclusion criteria presented with a proximal left anterior descending artery in the EPO-AMI-I study. This type of STEMI patient will receive more benefit from adjunctive therapy [23, 24]. Second, we have shortened the therapeutic time window from the onset of chest pain to reperfusion time (from 24 h to 12 h), which will also result in a shorter time window between EPO administration and the onset of chest pain. For example, in rats with a permanent coronary occlusion, EPO does not effectively reduce myocardial infarct size when administered ≥24 h after the MI [25]. These protocol modifications of the EPO-AMI-I study will improve the efficacy and safety of low-dose EPO in patients with STEMI.

Safety of EPO in STEMI patients

In the EPO-AMI-I (12,000 IU) and EPOC studies (6,000 IU × 3) in which low-dose EPO was administered, the risk of cardiovascular events was not increased [11, 12]. When high-dose EPO was administered, the results were inconsistent. In the REVEAL study, the subanalysis showed that EPO (60,000 IU) had a higher incidence of serious adverse events, although the authors noted that the analysis was based on a very small number of events. Conversely, in

the HEBEIII study, the subanalysis revealed that EPO showed a trend toward a reduction of enzymatic infarct size and significantly reduced the incidence of the combined endpoint (cardiovascular death, myocardial infarction, instent thrombosis, unstable angina and heart failure). In the REVIVAL study, EPO (33,000 IU × 3) showed a trend toward an increased rate of serious adverse effects. Their meta-analysis showed that the administration of EPO appeared to be safe for patients with acute STEMI [26]. For the safety of patients in the EPO-AMI-II study, a report system for serious adverse events has been established, and the clinical research coordinator will often visit the hospitals that participate in this study. Recently, the post-hoc analysis suggested the association of high-dose EPO with the restenosis of the culprit lesion, although no significant differences in late lumen loss between the EPO and placebo groups were observed [27, 28]. Additionally, no significant difference in late lumen loss was found when low-dose EPO was used [11, 12].

Quantification of LV function and infarct size

In the EPO-AMI-II study, we are only including patients with a first STEMI because ECG-gated SPECT allows for no distinction between previous and new infarcts. The primary end point of this study is to evaluate the improvement of LVEF between the acute and chronic stages (Table 2). In the chronic stage, ECG-gated SPECT with adenosine allows for the evaluation of the residual myocardial ischemia. One alternative evaluation method is cardiac magnetic resonance imaging, which may be able to assess the at-risk area and the final infarct size, but this technique remains to be validated for quantification [29].

Conclusions

Because the randomized control trials conducted to date have used high-dose EPO and demonstrated heterogeneous results, the EPO-AMI-II study will clarify the safety and efficacy of low-dose EPO in STEMI patients with LV dysfunction in double-blind, placebo-controlled, multicenter studies (Appendix).

Appendix

Principle Investigators:
Aizawa Y, Komuro I.
Steering Committee:
Higo S, Ito H, Kato K, Kobayashi N, Ozawa T, Minamino T, Suzuki H. Tobaru T, Toba K.
Data Management:
Higo S, Takahara S (Medical Center for Translational Research Osaka University Hospital, Suita, Japan)
Statistical Analysis:
Yamamoto K, Hamasaki T (Department of Biomedical Statistics, Osaka University Graduate School of Medicine, Suita, Japan)
SPECT Core Center:
Ishida Y (Kansai Rosai Hospital Cardiovascular Center, Amagasaki, Japan), Sakata Y, (Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Japan), Yoshimura N (Department of Radiology, Niigata University Medical and Dental Hospital, Niigata, Japan)
Data and Safety Monitoring Board:
Fujio Y (Laboratory of Clinical Science and Biomedicine, Graduate School of Pharmaceutical Sciences, Osaka University, Japan), Hasegawa S (Department of Cardiology, Osaka Kosei Nenkin Hospital, Osaka, Japan), Seki Y (Department of Internal Medicine (Hematology), Niigata Prefectural Shibata Hospital, Japan). Sugimoto T (Department of Mathematical Sciences, Hirosaki University Graduate School of Science and Technology, Japan)
EPO-AMI-II Investigators:
Chiba-Nishi General Hospital, Chiba, Japan.
Yoshida T, Kuramochi M
Dokkyo University School of Medicine, Tochigi, Japan;
Ishimitsu T, Kobayashi N
Fujisawa City Hospital, Fujisawa, Japan
Yano H, Himeno H
Higashi-Osaka City General Hospital, Osaka, Japan
Kijima Y, Ichikawa M

Kansai Rosai Hospital, Amagasaki, Japan;
Uematsu M, Watanabe T
Kanto Rosai Hospital, Kawasaki, Japan
Namiki A, Shibata M
Kokura Memorial Hospital, Kitakyushu, Japan
Yokoi H, Nomura A
National Cerebral and Cardiovascular Center, Suita, Japan
Yasuda S, Kotani J
Niigata University Medical and Dental Hospital, Niigata, Japan;
Toba K, Ozawa T
Nippon Medical School, Tokyo, Japan
Mizuno K, Yasutake M
Nozaki Tokushukai Hospital, Osaka, Japan
Okutsu M, Sumitsuji S
Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan;
Ito H, Kusano K
Osaka General Medical Center, Osaka, Japan;
Fukunami M, Yamada T
Osaka National Hospital, Osaka, Japan;
Yasumura Y, Hirooka K
Osaka Police Hospital, Osaka, Japan;
Ueda Y, Hirata A
Osaka Rosai Hospital, Sakai, Osaka, Japan;
Nishino M, Makino N
Osaka University Graduate School of Medicine, Suita, Japan;
Komuro I, Minamino T
Sakakibara Heart Institute, Fuchu, Japan
Tobaru T, Asano R
Senri Critical Care Medical Center, Osaka Saiseikai Senri Hospital, Suita, Japan
Ito N, Doi, Y
Shonan Kamakura General Hospital, Kamakura, Japan
Saito S, Tobita K
Showa University Fujigaoka Hospital, Yokohama, Japan;
Suzuki H, Wakabayashi K
Showa University School of Medicine, Tokyo, Japan
Kobayashi Y, Koba S
St. Marianna University School of Medicine, Kawasaki, Japan
Yoneyama Y, Akashi Y
Tachikawa General Hospital, Nagaoka, Niigata, Japan;
Aizawa Y, Sato M

References

1. Lloyd-Jones D, Adams RJ, Brown TM, et al. Executive summary: heart disease and stroke statistics–2010 update: a report from the American Heart Association. Circulation. 2010;121:948–54.

2. Takii T, Yasuda S, Takahashi J, et al. Trends in acute myocardial infarction incidence and mortality over 30 years in Japan: report from the MIYAGI-AMI Registry Study. Circ J. 2010;74:93–100.
3. Sutton MG, Sharpe N. Left ventricular remodeling after myocardial infarction: pathophysiology and therapy. Circulation. 2000;101:2981–8.
4. Minamino T. Cardioprotection from ischemia/reperfusion injury: basic and translational research. Circ J. 2012;76:1074–82.
5. Jelkmann W. Erythropoietin: structure, control of production, and function. Physiol Rev. 1992;72:449–89.
6. Hirata A, Minamino T, Asanuma H, et al. Erythropoietin just before reperfusion reduces both lethal arrhythmias and infarct size via the phosphatidylinositol-3 kinase-dependent pathway in canine hearts. Cardiovasc Drugs Ther. 2005;19:33–40.
7. Hirata A, Minamino T, Asanuma H, et al. Erythropoietin enhances neovascularization of ischemic myocardium and improves left ventricular dysfunction after myocardial infarction in dogs. J Am Coll Cardiol. 2006;48:176–84.
8. Ott I, Schulz S, Mehilli J, et al. Erythropoietin in patients with acute ST-segment elevation myocardial infarction undergoing primary percutaneous coronary intervention: a randomized, double-blind trial. Circ Cardiovasc Interv. 2010;3:408–13.
9. Voors AA, Belonje AM, Zijlstra F, et al. A single dose of erythropoietin in ST-elevation myocardial infarction. Eur Heart J. 2010;31:2593–600.
10. Najjar SS, Rao SV, Melloni C, et al. Intravenous erythropoietin in patients with ST-segment elevation myocardial infarction: RE-VEAL: a randomized controlled trial. JAMA. 2011;305:1863–72.
11. Ozawa T, Toba K, Suzuki H, et al. Single-dose intravenous administration of recombinant human erythropoietin is a promising treatment for patients with acute myocardial infarction—randomized controlled pilot trial of EPO/AMI-1 study. Circ J. 2010;74:1415–23.
12. Taniguchi N, Nakamura T, Sawada T, et al. Erythropoietin prevention trial of coronary restenosis and cardiac remodeling after ST-elevated acute myocardial infarction (EPOC-AMI): a pilot, randomized, placebo-controlled study. Circ J. 2010;74:2365–71.
13. Opie LH. Erythropoietin as a cardioprotective agent: down but not out. Heart. 2011;97:1537–9.
14. Vaziri ND. Thrombocytosis in EPO-treated dialysis patients may be mediated by EPO rather than iron deficiency. Am J Kidney Dis. 2009;53:733–6.
15. Baker JE, Kozik D, Hsu AK, Fu X, Tweddell JS, Gross GJ. Darbepoetin alfa protects the rat heart against infarction: dose-response, phase of action, and mechanisms. J Cardiovasc Pharmacol. 2007;49:337–45.
16. Uesaka H. A personal view of methods of sample size estimation for a clinical trial. Jpn J Biom. 2003;24:17–41.
17. Jennison C, Turnbull BW. Group sequential methods with applications to clinical trials. Boca Raton: Chapman & Hall/CRC; 2000.
18. Zhang J, Somani AK, Siminovitch KA. Roles of the SHP-1 tyrosine phosphatase in the negative regulation of cell signalling. Semin Immunol. 2000;12:361–78.
19. Kanellakis P, Pomilio G, Agrotis A, et al. Darbepoetin-mediated cardioprotection after myocardial infarction involves multiple mechanisms independent of erythropoietin receptor-common beta-chain heteroreceptor. Br J Pharmacol. 2010;160:2085–96.
20. Hausenloy DJ, Baxter G, Bell R, et al. Translating novel strategies for cardioprotection: the Hatter Workshop Recommendations. Basic Res Cardiol. 2010;105:677–86.
21. Schwartz Longacre L, Kloner RA, Arai AE, et al. New horizons in cardioprotection: recommendations from the 2010 National Heart, Lung, and Blood Institute Workshop. Circulation. 2011;124:1172–9.
22. Downey JM, Cohen MV. Why do we still not have cardioprotective drugs? Circ J. 2009;73:1171–7.
23. Piot C, Croisille P, Staat P, et al. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. N Engl J Med. 2008;359:473–81.
24. Botker HE, Kharbanda R, Schmidt MR, et al. Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: a randomised trial. Lancet. 2010;375:727–34.
25. Moon C, Krawczyk M, Paik D, Lakatta EG, Talan MI. Cardioprotection by recombinant human erythropoietin following acute experimental myocardial infarction: dose response and therapeutic window. Cardiovasc Drugs Ther. 2005;19:243–50.
26. Li J, Xu H, Gao Q, Wen Y. Effect of erythropoiesis-stimulating agents in acute ST-segment elevation myocardial infarction: a systematic review. Eur J Clin Pharmacol. 2011;68:469–77.
27. Stein A, Mohr F, Laux M, et al. Erythropoietin-induced progenitor cell mobilisation in patients with acute ST-segment-elevation myocardial infarction and restenosis. Thromb Haemost. 2012;107:769–74.
28. Minamino T, Toba K, Higo S, Nakatani D, Ozawa T. Erythropoietin, progenitor cells and restenosis. A critique of Stein et al. Thromb Haemost. 2012;107:1193.
29. Carlsson M, Ubachs JF, Hedstrom E, Heiberg E, Jovinge S, Arheden H. Myocardium at risk after acute infarction in humans on cardiac magnetic resonance: quantitative assessment during follow-up and validation with single-photon emission computed tomography. JACC Cardiovasc Imaging. 2009;2:569–76.

Complement C1q Activates Canonical Wnt Signaling and Promotes Aging-Related Phenotypes

Atsuhiko T. Naito,[1,3] Tomokazu Sumida,[4] Seitaro Nomura,[4] Mei-Lan Liu,[4] Tomoaki Higo,[1] Akito Nakagawa,[1] Katsuki Okada,[1] Taku Sakai,[1] Akihito Hashimoto,[1] Yurina Hara,[1] Ippei Shimizu,[4] Weidong Zhu,[4] Haruhiro Toko,[4] Akemi Katada,[4] Hiroshi Akazawa,[1,3] Toru Oka,[1,3] Jong-Kook Lee,[1,3] Tohru Minamino,[4] Toshio Nagai,[4] Kenneth Walsh,[5] Akira Kikuchi,[2] Misako Matsumoto,[6] Marina Botto,[7] Ichiro Shiojima,[1,3] and Issei Komuro[1,3,4,*]

[1]Department of Cardiovascular Medicine
[2]Department of Molecular Biology and Biochemistry
Osaka University Graduate School of Medicine, Osaka 565-0871, Japan
[3]Japan Science and Technology Agency, CREST, Tokyo 102-0075, Japan
[4]Department of Cardiovascular Science and Medicine, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan
[5]Molecular Cardiology, Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, MA 02118, USA
[6]Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Hokkaido 060-8638, Japan
[7]Centre for Complement and Inflammation Research, Department of Medicine, Imperial College London, London SW7 2AZ, UK
*Correspondence: komuro-tky@umin.ac.jp
DOI 10.1016/j.cell.2012.03.047

SUMMARY

Wnt signaling plays critical roles in development of various organs and pathogenesis of many diseases, and augmented Wnt signaling has recently been implicated in mammalian aging and aging-related phenotypes. We here report that complement C1q activates canonical Wnt signaling and promotes aging-associated decline in tissue regeneration. Serum C1q concentration is increased with aging, and Wnt signaling activity is augmented during aging in the serum and in multiple tissues of wild-type mice, but not in those of C1qa-deficient mice. C1q activates canonical Wnt signaling by binding to Frizzled receptors and subsequently inducing C1s-dependent cleavage of the ectodomain of Wnt coreceptor low-density lipoprotein receptor-related protein 6. Skeletal muscle regeneration in young mice is inhibited by exogenous C1q treatment, whereas aging-associated impairment of muscle regeneration is restored by C1s inhibition or C1qa gene disruption. Our findings therefore suggest the unexpected role of complement C1q in Wnt signal transduction and modulation of mammalian aging.

INTRODUCTION

Wnts constitute a large family of secreted proteins that elicit evolutionarily conserved intracellular signaling and affect diverse cellular responses during development. Wnt signaling also plays critical roles in various physiological and pathological processes in adult organisms, including stem cell self-renewal/differentia-

tion, degenerative diseases, and carcinogenesis (Blanpain et al., 2007; Clevers, 2006; Logan and Nusse, 2004). The β-catenin-dependent canonical Wnt pathway is the most understood signaling cascade initiated by Wnt proteins. Upon Wnt stimulation, cytosolic β-catenin is stabilized and translocates to the nucleus, where it binds to T cell factor/Lymphoid enhancer factor (Tcf/Lef) and induces Tcf/Lef-dependent transcription (Logan and Nusse, 2004). This canonical Wnt signaling is mediated by two types of cell surface receptors, the Frizzled (Fz) family of serpentine proteins and the single-transmembrane protein low-density lipoprotein receptor-related protein 5/6 (LRP5/6) (Angers and Moon, 2009; MacDonald et al., 2009).

Recent studies have revealed a role of Wnt signaling in the regulation of mammalian aging. Wnt/β-catenin signaling is augmented in a mouse model of accelerated aging (Liu et al., 2007), and inhibition of canonical Wnt signaling reverses the aging-associated impairment of skeletal muscle regeneration (Brack et al., 2007). Moreover, this age-related activation of Wnt signaling was attributed to the substance(s) in the serum that binds to the extracellular cysteine-rich domain (CRD) of Fz (Brack et al., 2007). However, because Wnt proteins tightly bind to the cell surface and/or extracellular matrix and are thought to act in a short-range manner (Kikuchi et al., 2007; White et al., 2007), the substance(s) in the serum that activates Wnt signaling was assumed to be distinct from classical Wnt proteins.

Here, we show that complement C1q is an activator of Wnt signaling. C1q activates canonical Wnt signaling by binding to Fz receptors and subsequently inducing C1s-dependent cleavage of the ectodomain of LRP6. Serum C1q concentration and the expression of C1q in various tissues are increased with aging, which are associated with increased Wnt signaling activity in serum and in multiple tissues during aging. We further demonstrate that activation of Wnt signaling by C1q accounts for the

A

TOPFLASH

14 12 10 8 6 4 2 0

Serum (-) Mouse Serum Human Serum Wnt3A

B

TOPFLASH

12 10 8 6 4 2 0

Serum (-) Human Serum IgG/Fc Fz8/Fc

Human Serum

C

TOPFLASH

2.5 2 1.5 1 0.5 0

Young Aged

D

TOPFLASH

6 5 4 3 2 1 0

Wild Sham PO DCM

E

M.W.
250
150
100
75
50
37
25

Wild Sham PO DCM

F

Precipitation by Protein G   M.W.

IB human Fc

C1qa

≈37
≈25
≈25

426-kDa C1qa protein

IgG Fc IgG Fc + C1q Fz8 Fc Fz8 Fc + C1q

G

Precipitation by Nickel-agarose   M.W.

IB Myc

C1qa

≈75
≈50
≈25

AP His-Myc + C1q   AP Fz8 His-Myc + C1q

H

Binding curve

Specific Binding to Fz8 (nmol/mg)

8 7 6 5 4 3 2 1 0

0 5 10 15 20 25 30 35 40 45 50
(nM)

I

Scatchard plot

Bound/Free

0.6 0.5 0.4 0.3 0.2 0.1 0

0 0.2 0.4 0.6 0.8 1 1.2
Bound (nM)

J

TOPFLASH

14 12 10 8 6 4 2 0

C1q (μg/mL) (-) 50 100 100 + Fz8/Fc 100 + Dkk-1

K

Cytosolic Protein

β-catenin

actin

Control C1q

L

Relative Expression of Axin2 mRNA

40 30 20 10 0

Control C1q Wnt3A

M

Dose-Response Curve

x 10⁴

TOPFLASH (Firefly luciferase activity)

4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0

0.01 0.1 1 10 100 1000 10000
C1q concentration (nM)

Control
Fz8 overexpression

N

Dose-Response Curve

x 10⁵

TOPFLASH (Firefly luciferase activity)

3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0

0.001 0.01 0.1 1 10 100
Wnt3A concentration (nM)

Control
Fz8 overexpression