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Studies on Establishment of Cardiotoxicity Biomarkers in Rats Based on Toxicogenomic Technologies

（トキシコゲノミクス手法を用いたラットにおける心筋毒性バイオマーカーの構築に関する研究）

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Chapter 3 Identification of a plasma miRNA biomarker for chemically-induced cardiotoxicity in rats
For human health, producing safer drugs is a mission of pharmaceutical companies. In medical drug development, candidate drugs are initially evaluated for any toxicity potentials in preclinical studies using experimental animals, and then, the developmental phase can be proceeded to clinical studies in humans. Therefore, it is essential to detect the potential toxicity of candidate drugs during preclinical studies. Although there are a huge number of candidate drugs in the initial developmental phase, these candidate drugs are gradually prioritized and selected mainly from the viewpoint of efficacy and safety. Preclinical toxicity studies are conducted several times depending on their developmental phase; they are usually started with short studies, and then gradually followed by longer ones. Therefore, detecting toxicity at the earliest possible stage can lead to a reduction in overall costs and labor requirements during novel drug development, obviously along with reducing toxicity events in humans.

Cardiotoxicity has a major impact on the development of novel drugs due to the lethality. In fact, cardiovascular problems are one of the main safety reasons accounting for drug withdrawal from the USA and EU markets (McNaughton et al., 2014; Qureshi et al., 2011). Classical methods to assess cardiotoxicity in preclinical studies are histopathological examination of the heart tissue and detection of plasma biomarkers such as aspartate transaminase (AST), lactate dehydrogenase (LDH), and creatine kinase (CK) (Boyd, 1988; Loeb and Quimby, 1999). These evaluations have been successfully conducted for many years as adequately validated methods. However, there is still room for improvement in cardiotoxicity evaluation when considering more efficient selection of the prospective candidate drugs in the early
developmental phase. Histopathological examination is a great evaluation method which can clarify the nature and localization of toxicities; however, the method can only detect actualized changes such as degeneration, necrosis, or inflammation, and there is a weakness of insufficient predictive performance. Regarding plasma biomarkers such as AST, LDH, and CK, they are lacking in organ specificity; AST and LDH are known to be also distributed in the liver, hemocytes, and skeletal muscles, in addition to the heart; and CK is abundant in both the heart and skeletal muscles (Frank et al., 1978; Hoffman et al., 1999). Recently, plasma cardiac troponin (cTn) has been described as a useful plasma biomarker for cardiotoxicity in experimental animals and humans. Cardiac troponin I and T (cTnI and cTnT, respectively) are cardiotoxicity-specific biomarkers, and have been shown to be more sensitive than the classical plasma biomarkers (O'Brien, 2008; Reagan, 2010; Tonomura et al., 2009, 2012). However, plasma cTn shows transient elevation due to its rapid clearance, and the blood sampling time point therefore needs to be set appropriately for use in rats (Tonomura et al., 2012). Additionally, it has been reported that serum levels of cTn increase in patients with chronic kidney disease without cardiac symptoms (Ahmadi et al., 2014). Considering the disadvantages of these existing methods, it is evident that novel methods for evaluating cardiotoxicity are still required.

The goal of a series of studies was to establish novel genomic biomarkers for detecting cardiotoxicity in rats using toxicogenomics technologies. Toxicogenomic analysis has been used to determine the mechanisms underlying chemical toxicity and to aid in the prediction of the early toxic effects of a drug based on specific gene expression changes (Battershill, 2005; Heinloth et al., 2004; Irwin et al., 2004; Searfoss et al., 2005). On the other hand, this approach has been successfully applied in the
identification of genomic biomarkers that predict and detect chemically-induced toxicity (Fabre et al., 2009; Ge and He, 2009). Microarray technology can supply comprehensive gene expression data efficiently in a single analysis and it is considered to be a great tool for the detection of the most appropriate genomic biomarkers. In the current studies, the author selected rats as species for which novel biomarkers were established. Although toxicity evaluations must be conducted in both rodent and non-rodent species in preclinical studies, the evaluation using rodents is initially selected in the early phase of drug development due to a small amount of compound to be synthesized. Among rodents, rats are widely accepted and used throughout preclinical toxicity studies.

First, the author focused on the development of novel mRNA biomarkers for cardiotoxicity using rat heart tissues. In the onset of toxicity, alterations of gene expression profiles inevitably occur in the toxicity target organ, and some alterations of gene expressions probably precede obvious toxicity such as histopathological changes. Therefore, mRNA biomarkers using heart tissues were expected to be the most promising in predicting performance. In the first chapter, the author obtained comprehensive gene expression data of the heart in rats administered with several cardiotoxicity compounds using DNA microarray. Chemically-induced cardiotoxicity are caused by various mechanisms. The author aimed to explore mRNA biomarkers to be used broadly in the early developmental phase. Therefore, 33 genes which showed up-regulation in common with these compounds were identified as potential mRNA biomarkers. Additionally, the author confirmed that 5 of the 33 genes showed sustained up-regulation with high expression levels.

In the second chapter, the author aimed to construct a multi-gene biomarker
using an optimized support vector machine. A multi-gene biomarker can lead to more efficient evaluation than using each gene independently. Each gene consisting of a multi-gene biomarker should be explainable from the function of the gene product and the functional relationship between each gene was considered to be important. In the second chapter, 8 genes were re-selected from the 33 genes picked out in the first chapter, and diagnostic accuracy for the genes was determined by a receiver operating characteristic (ROC) analysis using more cardiotoxic and non-cardiotoxic compounds. Then, the author constructed an optimal multi-gene biomarker and compared its diagnostic accuracy to those of classical evaluation methods including cTnI in single and repeated dosing conditions.

In the third chapter, the author aimed to explore plasma microRNA (miRNA) biomarkers for cardiotoxicity in rats. Although the multi-gene model established in the second chapter is promising for use in preclinical phase, it cannot be applied to humans due to its invasive nature. In drug development, biomarkers which can be applied to both experimental animals and humans are ideal in order to monitor toxicity throughout a whole of developmental and post-marketing phases. The existence of miRNAs in circulating blood has been reported, and some circulating (plasma/serum) miRNAs have been proposed as non-invasive, stable biomarkers for various pathological conditions (Haider et al., 2014). It was considered that the ideal miRNA for plasma biomarkers should be abundantly and exclusively produced in the target organ, since large amounts of such a miRNA would then leak into circulating blood from the cytoplasm of the injured cells of the target organ. The author selected miRNAs which were abundantly and exclusively expressed in the heart tissue using a comprehensive miRNA microarray dataset in normal rats (Minami et al., 2014). Then,
the usefulness of selected miRNAs as plasma biomarkers was confirmed in rat cardiotoxicity under single and repeated dosing conditions.

The results of a series of studies indicate the superiority of the novel genomic biomarkers in comparison with classical evaluation methods such as histopathological examination and plasma cTn measurement. These novel tools will assist to prioritize candidate drugs more efficiently and to produce safer drugs in the future.
Chapter 1  Identification of potential genomic biomarkers for chemically-induced cardiotoxicity in rats
Introduction

In the onset of toxicity, alterations of gene expression profiles are induced in the toxicity target organ. Toxicogenomic analysis has been suggested as an appropriate tool for the determination of the mechanisms underlying chemically-induced toxicity and to aid in the prediction of the early toxic effects of a drug (Battershill, 2005; Heinloth et al., 2004; Irwin et al., 2004; Searfoss et al., 2005). For example, to detect genomic biomarkers for nephrotoxicity, a large number of studies have been conducted on various nephrotoxic animal models treated with prototypical compounds (Decristofaro and Daniels, 2008; Kondo et al., 2009; Thukral et al., 2005). Using this approach, genes such as kidney injury molecule 1 (Kim1), lipocalin 2 (Lcn2) and secreted phosphoprotein 1 (Spp1) were successfully identified as promising genomic biomarkers for acute nephrotoxicity in rats (Wang et al., 2008).

As for cardiotoxicity, several recent publications have described gene expression profiles using microarray analysis on heart tissues injured by cardiotoxic compounds. The hearts of rats treated with isoproterenol demonstrated alterations in gene expression related to necrosis and apoptosis, adaptation to hypertension, fatty acid metabolism, fetal genes and inflammation (Mikaelian et al., 2008), while genes related to metabolism, oxidative stress response, signal transduction, apoptosis and cardiac muscle structure were altered in the doxorubicin-treated mouse heart (Yi et al., 2006). Although these reports revealed that several genes are involved in the development of cardiotoxicity, these genes were only altered following treatment with each single compound. Information regarding genomic biomarkers for cardiotoxicity is still limited. In the chapter 1, the author aimed to identify potential genomic biomarkers
for early detection of cardiotoxicity in rats.

In this chapter, three cardiotoxic compounds, isoproterenol, doxorubicin and carbofuran, were chosen because of their different predominant pharmacological/toxicological mechanisms of action. Isoproterenol, a synthetic catecholamine used as a bronchodilation drug, is known to be a representative cardiotoxic compound that induces cardiac injury in rats (Mikaelian et al., 2008; Zhang et al., 2008). Doxorubicin is a member of the anthracycline anti-tumor drug family that has the potential cardiotoxicity in humans (Ferreira et al., 2008). Several acute and chronic animal models of cardiotoxicity have also been reported using doxorubicin (Iqbal et al., 2008; Kelishomi et al., 2008; Li et al., 2006). Carbofuran is a potent, reversible cholinesterase inhibitor that has been reported to cause cardiac injury in rats (Gupta et al., 1991).

In medical drug development, ideal biomarkers can be commonly applied to various compounds without relation to their toxicity mechanisms. Therefore, the author focused on genes which commonly showed alterations of their expressions in cardiotoxicity caused by the different compounds. Each of the three cardiotoxic compounds was administered to rats, and microarray data analysis was conducted at two time points using heart tissues. The expression patterns of the altered genes were also evaluated using hierarchical clustering analysis and principal component analysis (PCA), and the commonly up-regulated genes were characterized using gene ontology (GO) analysis. The data of microarray analysis obtained in this chapter will be a great help for exploring genomic biomarkers for cardiotoxicity in rats.
Materials and Methods

Chemical compounds:

Isoproterenol hydrochloride (Sigma-Aldrich, St Louis, MO, USA), doxorubicin hydrochloride (Kemprotech Ltd., Middlesbrough, UK) and carbofuran (Sigma-Aldrich) were used. Hereinafter, isoproterenol and doxorubicin are referred to by their free form names, and the dose levels are referred to as their free forms.

Animal and housing conditions:

Male Sprague-Dawley rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The animals were housed under a controlled temperature (20 – 26°C), humidity (30 – 70%), and light cycle (lights from 8 a.m. until 8 p.m.). All animals were allowed free access to food (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and water (sterilized city water via an automatic watering system with watering nozzles). The animals were 6 weeks of age at dosing.

Animal experimental design:

All experimental procedures were conducted after the study was approved by the Institutional Animal Care and Use Committee.

The animals were allocated into the following six groups containing 6 rats each: (i) control group [saline alone; subcutaneous injection (s.c.)]; (ii) isoproterenol-treated
group (0.01 mg/kg; s.c.); (iii) control group [saline alone; intraperitoneal injection (i.p.)]; (iv) low-dose doxorubicin-treated group (20 mg/kg; i.p.); (v) high-dose doxorubicin-treated group (40 mg/kg; i.p.); (vi) carbofuran-treated group (1.5 mg/kg; i.p.). Each chemical powder was dissolved in physiological saline at the required concentration immediately prior to dosing, and each solution was administered at 5 (s.c.) or 10 (i.p.) mL/kg. Animals were sacrificed 8 or 24 h after a single dose of each compound (n=3/time point). At necropsy, each animal was euthanized via exsanguination by cutting both the abdominal aorta and vena cava under pentobarbital sodium anesthesia. The hearts were then removed for histopathological examination and gene expression analysis.

**RNA extraction:**

Immediately after euthanasia, approximately 90 mg of tissue was obtained from the cardiac apex to the central portion of the left ventricle of each animal. The samples were stored in RNAlater® (Ambion, Austin, TX, USA) at −80°C until analyzed. The tissue samples were then thawed and homogenized in QIAzol Lysis Reagent with a TissueLyser (Qiagen, Valencia, CA, USA), and the total RNA extracted using the RNeasy® Mini Kit (Qiagen). The RNA concentration was determined using a NanoDrop ND1000 spectrophotometer (Labtech International, East Sussex, UK). RNA quality was verified using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

**Histopathological examination:**
After sampling the portion for RNA extraction, the hearts were fixed in 10% neutral buffered formalin, dehydrated in alcohol and embedded in paraffin. After paraffin sections were prepared and stained with hematoxylin and eosin (H&E) using a routine method, all samples were examined under a light microscope.

**Microarray analysis:**

Microarray analysis was undertaken using One-Cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, CA, USA) for cDNA synthesis, purification and synthesis of biotin-labeled cRNA according to the manufacturer’s instructions. Briefly, 10 μg of fragmented cRNA was hybridized to a Rat Genome 230 2.0 Array (Affymetrix) for 17 h at 45°C at 60 rpm. Then, the array was washed and stained with streptavidin-phycoerythrin using the Fluidics Station 450 (Affymetrix) and scanned with a Gene Array Scanner GCS3000 7G (Affymetrix). Microarray Analysis Suite 5.0 (MAS; Affymetrix) was used to quantify microarray signals and the intensities normalized for each chip.

**Microarray data analysis:**

Based on the statistical criteria outlined below, significant alterations in gene expression levels that were common to the treatment of isoproterenol, doxorubicin and carbofuran were extracted. As for doxorubicin, the data for rats dosed at 40 mg/kg, showing histopathological changes, were used in the process of gene selection. First,
the data was imported into the Spotfire® DecisionSite for Functional Genomics (Spotfire, Göteborg, Sweden), and all signal intensities for each array were normalized to the average value of all genes. Comparisons between each treated and corresponding control group at each time point were performed. Probe sets that were up-regulated more than twofold or down-regulated to less than half, and with a statistical significance of p<0.05 as estimated by the Student's t-test at one or both time points in common with two or all compounds were selected. The Affymetrix Detection Call algorithm was used for this step, and the following probe sets were eliminated: probe sets showing an absent call in all samples of the treated group when selecting up-regulated genes, and probe sets showing an absent call in all samples of control when selecting down-regulated genes.

To increase the confidence in the analysis of microarray data, only probe sets with highly reliable annotation (Affymetrix's grade A or B annotation) were selected for further analysis.

Using the list of extracted genes, hierarchical clustering analysis was conducted in order to visualize the time course changes of gene expression, and in particular, to further extract candidates commonly altered in the heart tissue of rats treated with isoproterenol, high-dose doxorubicin or carbofuran. This analysis was also applied for the low and high dose levels of doxorubicin in order to investigate the extent of up-regulation of these genes at a dose that did not result in histopathological changes. In hierarchical clustering, base-2 logarithmic transformation was applied to gene expression data. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method and Euclidean distance similarity measure were used for the analysis. PCA of the selected probe sets was also applied in all individual data including the low-dose doxorubicin group. For PCA, normalized signal values corrected by the
Z-score were used.

**Gene ontology (GO) analysis:**

GO analysis was performed using the DAVID Functional Annotation tool (Database for Annotation, Visualization, and Integrated Discovery; [http://david.abcc.ncifcrf.gov/](http://david.abcc.ncifcrf.gov/)). A p value of < 0.005 as determined by Fisher’s exact test was considered statistically significant. Level 5 analysis was adopted for the term of the GO biological process.

**Quantitative real-time RT-PCR:**

Expression levels of *Spp1*, *Fhl1*, *Timp1*, *Ccl7*, and *Reg3b* were measured using one-step quantitative real-time PCR for all treatment groups. Total RNA (0.4 µg) was used as the template, and TaqMan Gene Expression Assays (for *Spp1*, assay ID Rn01449972_m1; *Fhl1*, assay ID Rn01402101_m1; *Timp1*, assay ID Rn00587558_m1; *Ccl7*, assay ID Rn01467286_m1; *Reg3b*, assay ID Rn00583920_m1) or TaqMan Endogenous Controls (for *Gapdh*, predesigned assay reagent applied by ABI, Applied Biosystems, Foster City, CA, USA) were employed as gene-specific probe and primer sets. Quantitative RT-PCR was performed using QuantiTect™ Probe RT-PCR Kit (Qiagen), and transcript level was quantitated with ABI PRISM 7500 Fast System (Applied Biosystems) according to the manufacturer’s instructions. Reverse transcription and amplification conditions were as follows: 50°C for 30 min, 95°C for 15 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The resulting cycle
threshold (Ct) value was processed based on the comparative Ct method, where Gapdh was used as an endogenous reference gene to normalize the expression level of target genes.
Results

Histopathology:

The results of histopathological examination are summarized in Table 1 and Figure 1. Isoproterenol- or carbofuran-treated animals demonstrated slight necrosis in the myocardium of the left ventricle and/or the septum through 8 and 24 h after treatment. This necrosis was accompanied by infiltration of inflammatory cells at both time points. In the hearts of all animals treated with doxorubicin at 40 mg/kg, edema in the interstitium was observed only at 24 h after treatment. These changes were not accompanied by necrosis or inflammatory lesions. There were no compound-related lesions in the animals treated with doxorubicin at a dose level of 20 mg/kg.
Commonly altered genes following treatment with isoproterenol, doxorubicin and carbofuran:

Based on the statistical criteria, 36 probe sets (33 genes) were selected as commonly up-regulated genes following isoproterenol, doxorubicin and carbofuran treatment (Table 2). In contrast, any commonly down-regulated genes were not detected and the author concluded that there was not a single promising candidate
biomarker in the down-regulated gene group. Therefore, the up-regulated genes were focused on in the further analysis.

Using hierarchical clustering, the time point showing significant gene up-regulation was not consistent for the three compounds. For the isoproterenol and carbofuran treatment groups, the extracted 36 probe sets were mainly up-regulated at 8 h after treatment, while up-regulation was predominantly detected at 24 h for the doxorubicin (40 mg/kg) treatment group. The vast majority of genes showed transient up-regulation only at 8 or 24 h after treatment under this study condition. However, genes such as Spp1, Fhl1, Timp1, Ccl7 and Reg3b showed a relatively continuous and high up-regulation at both time points (Figure 2A).

The treatment group receiving 20 mg/kg of doxorubicin, which did not demonstrate any histopathological lesions, also revealed up-regulation of many genes. The up-regulation level observed at 8 h after treatment was similar to the level observed following treatment at 40 mg/kg. However, the gene expression level at 20 mg/kg was found to drop to a level lower than those following treatment with 40 mg/kg at 24 h after treatment (Figure 2B).
Principal component analysis (PCA) of the selected probes:

As shown in Figure 3, PCA was performed on all animals using the expression data of the selected 36 probe sets. Treated samples were separated from controls toward the first principal component (PC1) direction for at least one time point (69.4%). In
order to examine the dose-dependency on gene expression in the doxorubicin-treated groups, the author compared the data of the 20 mg/kg group to that of the 40 mg/kg group. At 8 h after treatment, the PC1 values of the animals in both groups were almost the same level, despite the observation that the animals in the 20 mg/kg treatment group showed no histopathological lesions. At 24 h following treatment, the PC1 values demonstrated a dose-related increase in gene expression caused by administration of doxorubicin.

**Functional classification of selected gene sets:**

GO analysis was performed on the selected 36 probe sets in order to determine the biological profile. The representative categories of biological processes detected in the GO analysis were summarized in Table 3.

<table>
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<th>Term</th>
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<tr>
<td>Chemotaxis</td>
<td>15.79%</td>
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<tr>
<td>Positive regulation of cell proliferation</td>
<td>13.16%</td>
</tr>
<tr>
<td>Cell part morphogenesis</td>
<td>13.16%</td>
</tr>
<tr>
<td>Cell projection organization and biogenesis</td>
<td>13.16%</td>
</tr>
<tr>
<td>Neuron development</td>
<td>13.16%</td>
</tr>
<tr>
<td>Cell projection morphogenesis</td>
<td>13.16%</td>
</tr>
<tr>
<td>Tissue regeneration</td>
<td>10.53%</td>
</tr>
<tr>
<td>Leukocyte migration</td>
<td>7.89%</td>
</tr>
<tr>
<td>Leukocyte chemotaxis</td>
<td>7.89%</td>
</tr>
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</table>
Quantitative real-time RT-PCR of the selected five genes:

Quantitative real-time RT-PCR was conducted on *Spp1, Fhl1, Timp1, Ccl7* and *Reg3b* and resulted in relatively consistent findings to that generated by the microarray analysis. As shown in Figure 4, almost all of the five genes demonstrated prominent up-regulation and showed a clear difference from the control group at both time points following treatment with the three compounds. Even in the doxorubicin 20 mg/kg group without any histopathological lesions, several genes showed reliable up-regulation at each time point.
Discussion

In the chapter 1, the author identified commonly up-regulated genes in the rat heart following treatment with isoproterenol, doxorubicin or carbofuran using a toxicogenomics approach. Until now, cellular and molecular mechanisms involved in cardiotoxicity by these chemicals have been identified. As for isoproterenol, increasing evidence has indicated that reactive oxygen species cause mitochondrial death and that β-adrenergic receptor stimulation leads to cardiomyocyte apoptosis (Remondino et al., 2003). Doxorubicin cardiotoxicity is thought to be due to oxidative stress and DNA damage (Berthiaume and Wallace, 2007; L’Ecuyer et al., 2006). Carbofuran toxicity is thought to be an indirect effect, possibly mediated by the release of non-physiological amounts of endogenous catecholamines (Singer et al., 1987; Tryphanas et al., 1996), while the precise mechanisms have not been explored. In spite of these differences, microarray analysis revealed that 33 genes involved in various physiological functions were commonly induced in the rat heart at the early stage of cardiotoxicity.

From GO analysis, it was detected that the selected genes were functionally associated with chemotaxis of inflammatory cells or tissue regeneration. For example, the author identified chemokine (C-C motif) receptor 1 (Ccr1), which is one of the CC chemokine receptors involved in the recruitment of inflammatory cells during cardiotoxicity in mice (Kaya et al., 2008; Liehn et al., 2008). The author also identified Spp1, a gene up-regulated during cardiotoxicity and that has been reported to be involved in cardiac fibrosis (Zahradka, 2008). Versican (Vcan) is induced in the infarcted rat heart, and is associated with monocyte infiltration and increased extracellular matrix
These genes appear to be strongly related with the inflammation and regeneration processes that occur following myocardial injury.

The appearance of the histopathological lesions is different among the treated groups. In isoproterenol- or carbofuran-treated rats, slight necrosis of myocardium and infiltration of inflammatory cells were observed. In contrast, the doxorubicin 40 mg/kg-treated rats showed interstitial edema without necrotic or inflammatory lesions. Despite these differences in the appearance of the histopathological lesions, similar gene alterations related to inflammation and regeneration processes were up-regulated in both the isoproterenol-/carbofuran- and doxorubicin-treated rats. A previous study has reported that doxorubicin-induced cardiac injury was morphologically characterized by swollen muscle fibers with interstitial edema and inflammation (Osman et al., 2009). In addition, cardiac lesions including vacuolation of the sarcoplasmic reticulum and mitochondrial degeneration of cardiomyocyte and interstitial edema were found 4 days after the intraperitoneal injection of 20 mg/kg of doxorubicin (Doroshow et al., 1985). Therefore, it is thought that edema is the primary change which leads to more prominent lesions, and that inflammation might also be observed in the doxorubicin-treated group at later time points than 24 h after treatment. These findings were supported by the evidence that the up-regulation of the probe sets was mainly observed at 24 h after treatment in the doxorubicin 40 mg/kg group, while probe sets were mainly up-regulated at 8 h following isoproterenol or carbofuran treatment. Interestingly, a lot of genes showed up-regulation even in rats without any histopathological lesions after the treatment of doxorubicin at 20 mg/kg. This result encourages the usefulness of the selected genes as promising biomarkers which can detect minute cardiotoxicity without histopathological lesions. Such high sensitivities may lead to the high predictive
performance for detecting cardiotoxicity in preclinical studies.

In the early drug developmental phase, there are a lot of drug candidates with various toxicity and toxicokinetics profiles. Therefore, sustained up-regulation leads to high diagnostic accuracy of cardiotoxicity in preclinical studies. In fact, transient change is a weak point of plasma biomarkers for cardiotoxicity; cardiac troponin I (cTnI) and cardiac troponin T (cTnT) showed transient increase and they returned to the basal level by 24 h after cardiotoxic compound administration (Tonomura et al., 2009). Therefore, the author focused on the following five genes: \textit{Spp1}, \textit{Fhl1}, \textit{Timp1}, \textit{Ccl7}, and \textit{Reg3b}, that consistently showed high expressions.

\textit{Spp1}, whose protein product is known as osteopontin, a phosphorylated glycoprotein induced during inflammation and is known to be related to cardiotoxicity. Although osteopontin is not expressed in the un-stressed heart, it is increased under pathological conditions such as cardiac infarction in animal models (Komatsubara et al., 2003; Trueblood et al., 2001). \textit{SPP1} or its protein product is also up-regulated during human dilated cardiomyopathy (Stawowy et al., 2002; Satoh et al., 2005). During cardiac fibrosis, osteopontin functions in the differentiation of fibroblasts into myofibroblasts, and plays an important role in pathogenesis (Lenga et al., 2008). \textit{Fhl1} is a cytoskeletal protein enriched in the skeletal muscle and heart (Lee et al., 1998). Its expression is increased during human hypertrophic cardiomyopathy and hypertrophic or \(\beta\)-adrenergic-induced cardiomyopathy mouse models (Chu et al., 2000; Gaussin et al., 2003; Lim et al., 2001). It has also been reported that \textit{Fhl1} plays an important role in the development of hypertrophy via sensing of the biomechanical stress responses in the sarcomeres in mice (Sheikh et al., 2008). \textit{Timp1} is reported to be related to fibrosis during cardiac remodeling in mice, and to be up-regulated during heart failure in humans.
(Barton et al., 2003; Szalay et al., 2009). Protein products of *SPP1* and *TIMP1* have also been reported as a plasma biomarker during heart failure in humans (Milting et al., 2008). *Ccl7*, which is also known as *Mcp3*, is a member of the CC chemokine family and has been reported to be involved in fibrosis such as renal fibrosis, liver fibrosis and systemic sclerosis (Klein et al., 2009; Ong et al., 2003; Saha et al., 2007). *Ccl7* is also known to induce collagen synthesis in fibroblasts (Ong et al., 2009). Given these reports, it was suggested that *Ccl7* may be associated with collagen synthesis during the remodeling process after myocardium injury. *Reg3b* is also known as *Reg2* or pancreatitis-associated protein (*Pap1*) in the rat and HIP/PAP in humans (Tebar et al., 2008). *Pap1* has been reported to be up-regulated during rat autoimmune myocarditis (Watanabe et al., 2008). Although its precise role in cardiotoxicity remains unknown, *Reg3b* may function as a growth factor during cell proliferation associated with tissue repair from the reports about liver or nerve injury (Lieu et al., 2005; Averill et al., 2002).

In the chapter 1, these five genes were focused only from a viewpoint of sustained up-regulation. Further large-scale validation studies using several types of compounds would be required to extract the most useful genomic biomarkers. In conclusion, the 33 genes identified in this chapter proved promising candidates that would be helpful in the development of genomic biomarkers for cardiotoxicity in rats.
Summary

In the chapter 1, the author aimed to detect potential genomic biomarkers for cardiotoxicity in rats using a toxicogenomics approach. Cardiotoxicity was induced in rats using the three cardiotoxic compounds, isoproterenol, doxorubicin and carbofuran. Then, microarray analysis was conducted using heart tissues at 8 or 24 h after treatment of each compound. Using statistical analysis, the author extracted 36 probe sets (33 genes) up-regulated in common by the three cardiotoxic compounds. GO analysis revealed that these genes were functionally associated with chemotaxis of inflammatory cells or tissue regeneration. Many genes showed up-regulation even in rats without any histopathological changes and therefore, it was considered that these genes are possibly biomarkers with high predictive performance. In addition, the author confirmed that \textit{Spp1}, \textit{Fhl1}, \textit{Timp1}, \textit{Ccl7}, and \textit{Reg3b} especially revealed a sustained up-regulation with high expression levels at both time points for all three compounds. In conclusion, even though definitive validation studies are required for the establishment of their usefulness and reliability, the 33 genes extracted in this chapter may prove to be the promising candidate genomic biomarkers for cardiotoxicity in rats.
Figure 1. Histopathological changes in the myocardium. (A) The left ventricle of a control rat at 24 h after treatment. The section demonstrates normal myocardium structure. (B) The left ventricle of an isoproterenol-treated rat at 8 h after treatment. Necrosis of the myocardium (arrowheads) and infiltration of inflammatory cells (arrow) was observed. (C) The left ventricle of a doxorubicin 40 mg/kg-treated rat at 24 h after treatment. Interstitial edema (asterisks) was observed. (D) The left ventricle of a carbofuran-treated rat at 8 h after treatment. Infiltration of inflammatory cells (arrows) was observed.
Figure 2. Hierarchical clustering analysis of the selected 36 probe sets. Base-2 logarithmic transformation was applied to gene expression data. The color indicates up-regulation (red: 2) and down-regulation (blue: −2). (A) Selected genes were commonly up-regulated in the isoproterenol (ISO)-, doxorubicin (DXR) 40 mg/kg-, and carbofuran (CAF)-treated groups. Most genes were up-regulated at 8 h after treatment in the ISO and CAF group, and 24 h after treatment in the DXR 40 mg/kg group. Some genes showed sustained up-regulation. (B) The expression of 36 probe sets was similar between DXR 20 mg/kg and 40 mg/kg groups at 8 h after treatment. However, 40 mg/kg group showed higher expression than 20 mg/kg group at 24 h after treatment.
Figure 3. Score plot of the first principal component (PC1) for the control-, isoproterenol (ISO)-, doxorubicin (DXR)- and carbofuran (CAF)-treated groups. The values of each cardiotoxic compound-treated rat were separated from that of the controls toward PC1 direction (69.4%).
Figure 4. Gene expression changes of candidate genomic biomarkers by real-time RT-PCR in the left ventricular myocardium at 8 h (A) or 24 h (B) after a single administration of isoproterenol (ISO), doxorubicin (DXR: 20 and 40 mg/kg) or carbofuran (CAF). The data represent the mean difference ($\Delta \Delta Ct$) of each compound-treated group compared to control ($n=3$/group) ± standard deviation.
Chapter 2  Construction of a multi-gene biomarker for chemically induced-cardiotoxicity in rats
Introduction

In the chapter 1, the author identified 33 candidate genomic biomarkers for cardiotoxicity in rats using microarray technologies (Mori et al., 2010). These genes showed the common up-regulation among three different compounds and seemed to be potential genomic biomarkers that can be applied to preclinical studies for detecting cardiotoxicity in rats. When the efficient prioritization of candidate drugs is taken into consideration, it is ideal that the toxicity can be detected using a small set of biomarkers. In the chapter 2, the author aimed to construct a multi-gene biomarker using an optimized support vector machine (SVM). A multi-gene biomarker can lead to a more efficient evaluation than using each gene independently. In the chapter 1, 5 of the 33 genes were focused on from sustained up-regulation with high expression levels. However, each gene consisting of a multi-gene biomarker should be explainable from the function of the gene product and the functional relationship between each gene is considered to be important. In the chapter 2, the author re-selected 8 genes based on their functions from 33 genes picked out in the chapter 1. The genes evaluated in this chapter included secreted phosphoprotein 1 (Spp1), four and a half LIM domains protein 1 (Fhl1), TIMP metallopeptidase inhibitor 1 (Timp1), serine (or cysteine) peptidase inhibitor, clade E, member 1 (Serpine1), branched chain aminotransferase 1, cytosolic (Bcat1), LIM and cysteine-rich domains 1 (Lmcd1), Rho family GTPase 1 (Rnd1), and transforming growth factor beta 2 (Tgfb2). Five of the selected genes (Spp1, Timp1, Serpine1, Rnd1, and Tgfb2) are involved in the extracellular matrix (ECM) deposition and degradation (Barton et al., 2003; Briest et al., 2004; Chardin, 2006; Ghosh et al., 2010; Lenga et al., 2008; Nobes et al., 1998; Szalay et al., 2009;
Takeshita et al., 2004; Zahradka, 2008; Zaman et al., 2009). Apoptosis is known to play a major role in the cardiotoxicity mechanism, and Bcat1 has a key role in apoptosis (Amin et al., 2011; Arola et al., 2000; Bledsoe et al., 1997; Chen et al., 2007; Eden and Benvenisty, 1999). Up-regulation of Fhl1 and Lmcd1 has been shown to be related to cardiac hypertrophy (Bian et al., 2010; Chu et al., 2000; Frank et al., 2010; Gaussin et al., 2003; Lim et al., 2001).

Reliable biomarkers should be verified using many positive and negative compounds. In the chapter 2, the author confirmed diagnostic accuracy of each of the 8 candidate genomic biomarkers by a receiver operating characteristic (ROC) analysis using more cardiotoxic and non-cardiotoxic compounds. Then, an optimal multi-gene model was constructed by SVM in order to achieve the best diagnostic performance. Additionally, the diagnostic accuracy was compared between the novel multi-gene biomarker and plasma cTnI measurement. The author selected cTnI for comparison, since cTn is the most useful plasma biomarker for cardiotoxicity, and it has been previously demonstrated that cTnI is higher and longer lasting as compared to cTnT (Tonomura et al., 2009).

Predictive performance is quite essential for biomarkers used in preclinical studies, since candidate drugs are usually evaluated for their toxicity potentials in short studies especially in the early phase of drug development. In the chapter 1, many of candidate genomic biomarkers showed up-regulation prior to the apparent histopathological changes, and they seem to be promising as biomarkers with high predictive performance. Therefore, the author also examined the predictive performance of the newly constructed multi-gene biomarker under a repeated dosing condition.
Materials and Methods

Chemical compounds:

The cardiotoxic compounds used in this chapter included: isoproterenol hydrochloride (Sigma-Aldrich, St Louis, MO, USA), doxorubicin hydrochloride (Kemprotec Ltd., Middlesbrough, UK), metaproterenol hemisulfate (Nacalai Tesque, Inc., Kyoto, Japan), allylamine (Nacalai Tesque, Inc.), mitoxantrone dihydrochloride (LKT Laboratories, Inc., MN, USA), cyclophosphamide monohydrate (Sigma-Aldrich), cyclosporine A (Kemprotec Ltd.), and allyl alcohol (Wako Pure Chemical Industries, Ltd., Osaka, Japan). All of these compounds are known to cause cardiotoxicity in rats or other animals during a single or repeated dosing condition.

The non-cardiotoxic compounds (negative controls) used in this chapter included: thioacetamide (Sigma-Aldrich), aminogluthethimide (Bachem Bioscience, Inc., PA, USA), acetaminophen (Sigma-Aldrich), p-aminophenol (Nacalai Tesque, Inc.), α-naphthylisothiocyanate (Sigma-Aldrich), methapyrilene hydrochloride (Sigma-Aldrich), carbon tetrachloride (Wako Pure Chemical Industries, Ltd.), chloroform (Sigma-Aldrich), phenylbutazone (Sigma-Aldrich), nitrofurantoin (ICN Biomedicals, Inc., OH, USA), triamterene (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), and n-phenylanthranilic acid (Sigma-Aldrich). These compounds are known to either cause toxicity in the liver, kidney, or adrenal gland in rats or other animals during a single or repeated dosing condition. Verapamil hydrochloride (Wako Pure Chemical Industries, Ltd.) and aconitine (LKT Laboratories, Inc.), which are known as a calcium channel blocker and a sodium channel activator respectively, were used in order to
confirm whether or not the genomic biomarkers could distinguish between degenerative structural changes and electrocardiographic alterations. These compounds were treated as non-cardiotoxic compounds in this chapter. Hereinafter, all compounds are referred to by their free form names, and the dose levels are referred to as their free forms.

Animals and housing conditions:

Male Sprague-Dawley rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The animals were housed under a controlled temperature (20 – 26°C), humidity (30 – 70%), and light cycle (lights from 8 a.m. until 8 p.m.). All animals were allowed free access to food (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and water (sterilized city water via an automatic watering system with watering nozzles). The animals were 6 weeks of age at the initial dosing.

Animal experimental design:

All experimental procedures were conducted after the study was approved by the Institutional Animal Care and Use Committee. Treatment conditions are summarized in Table 1. Experiment (Exp.) 1 and Exp. 2 utilized a large number of reference cardiotoxic and non-cardiotoxic compounds in order to verify the suitability of the candidate genomic biomarkers for cardiotoxicity. As described in Table 1, animals (n=6/group, with 3 animals used for each sampling point) were treated with each compound a single time. Control animals were treated with the corresponding vehicle alone using the same
experimental design. Dose levels that could cause histopathological changes in rat hearts were selected for the cardiotoxic compounds, isoproterenol, doxorubicin, metaproterenol, and allylamine; and dose levels that could possibly cause cardiotoxicity without histopathological changes were selected for cyclophosphamide based on information in the literature or from data obtained in pilot studies. Maximally tolerable single dose levels for the cardiotoxic compounds, mitoxantrone, cyclosporine A, and allyl alcohol were determined based on information in the literature or from data obtained in pilot studies. For the non-cardiotoxic compounds, toxic dose levels for the target organs were selected based on information in the literature or from data obtained in pilot studies. Each 3 animals were necropsied at 8 and 24 h after dosing. Under pentobarbital sodium anesthesia, animals were euthanized prior to necropsy by cutting both the abdominal aorta and vena cava. Heart samples were collected for gene expression analysis and histopathological examination. Only in Exp. 1, blood samples (0.25 mL at each point) were collected for cTnI measurements via the tail vein at 2 and 4 h after the dosing in 3 animals that were subsequently necropsied at 24 h after dosing. Blood samples (2 mL at each point) were also obtained via the vena cava from all animals necropsied at 8 or 24 h after dosing. Na heparin was used as the anticoagulant for cTnI measurement.

Exp. 3 was conducted in order to confirm the predictive performance of the multi-gene biomarker under a repeated dosing condition using doxorubicin. The dose of doxorubicin was lower than that used in Exp. 1, since the dose level was considered to be maximally tolerable under the repeated dosing condition. Doxorubicin was administered to rats at 3 mg/kg/day for a single day (n=3) or 7 days (n=4). Control animals were given saline alone in the same manner. Single-treatment animals were necropsied at 24 h after dosing, while 7-day treatment animals were necropsied at 24 h
after the final dosing. Using the same method detailed for Exp. 1 and Exp. 2, heart samples were also obtained for the gene expression analysis and histopathological examinations. For the plasma cTnI measurement, another animals (n=4) were administered with doxorubicin at 3 mg/kg/day for 7 days, with blood samples (0.25 mL at each point) obtained via the tail vein at 2, 4, 8, and 24 h after the first dosing, and at 24 h after the final dosing. Control animals were treated in the same manner with the exception of being administered with saline alone. Na heparin was used as the anticoagulant for cTnI measurement.

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**RNA extraction:**

Immediately after euthanasia, approximately 90 mg of tissue was obtained from the heart of each animal from the cardiac apex to the central portion of the left ventricle. Tissue samples were stored at $-80^\circ$C in RNAlater® (Ambion, Austin, TX, USA) until analyzed. Frozen heart tissues were thawed and homogenized using a TissueLyser (Qiagen, Valencia, CA, USA) in QIAzol Lysis Reagent (Qiagen), and total RNA was then extracted using an RNeasy® Mini Kit (Qiagen). The RNA concentration was determined using a NanoDrop ND1000 spectrophotometer (Labtech International, East Sussex, UK). RNA quality was verified using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

**Quantitative real-time RT-PCR:**

Expression of *Spp1, Fhl1, Timp1, Serpine1, Bcat1, Lmcd1, Rnd1*, and *Tgfb2* was measured using a one-step quantitative real-time RT-PCR. In each reaction, 80 ng of total RNA from each aliquot was used as the template. Either the TaqMan Gene Expression Assays (*Spp1*, assay ID Rn01449972_m1; *Fhl1*, assay ID Rn01402101_m1; *Timp1*, assay ID Rn00587558_m1; *Serpine1*, assay ID Rn01481341_m1*; *Bcat1*, assay ID Rn00568471_m1*; *Lmcd1*, assay ID Rn01426297_m1*; *Rnd1*, assay ID Rn01761912_m1*; *Tgfb2*, assay ID Rn00579674_m1*) or the TaqMan Endogenous Controls (*Actb*) (predesigned assay reagent supplied by Applied Biosystems, Foster City, CA, USA) were used for the sets of the gene-specific probe and primer pairs. Quantitative RT-PCR was performed using a QuantiTect™ Probe RT-PCR Kit (Qiagen),
with transcript levels quantitated using an ABI PRISM 7900 Fast System (Applied Biosystems) in accordance with the manufacturer’s protocols. Reverse transcription and amplification conditions were as follows: 50°C for 30 min, 95°C for 15 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The resulting cycle threshold (Ct) value was determined based on the comparative Ct method, where Actb was used as the endogenous reference gene for the purpose of normalizing the expression level of the target genes.

**Histopathological examination:**

After sampling the portion for RNA extraction, the hearts were fixed in 10% neutral buffered formalin, dehydrated in alcohol and embedded in paraffin. After paraffin sections were prepared and stained with hematoxylin and eosin (H&E) using a routine method, all samples were examined under a light microscope.

**Plasma cTnI measurement:**

Plasma samples were separated by centrifugation of the blood at 1500×g for 15 min under refrigeration, and the resulting plasma specimens were stored frozen at −80°C pending analysis. Plasma cTnI levels were measured using a commercial ELISA kit for rat cTnI (high sensitivity rat cardiac troponin-I ELISA kit; Life Diagnostics, Inc., PA, USA). Each sample was analyzed in duplicate.

**ROC analysis and construction of a multi-gene biomarker:**
Diagnostic accuracy of each parameter was evaluated by a ROC analysis based on a 5-fold cross validation. Exceptionally, the data of plasma cTnI measurement was not applied to a 5-fold cross validation, since its large deviation could lead to a misunderstanding of the prediction performance under the 5-fold cross validation. An optimal multi-gene model was constructed by SVM, and evaluated by a ROC analysis in the same manner. In the 5-fold cross validation, the whole positive and negative data set were randomly divided into 5 subsets of roughly equal size, respectively. Then, each gene was trained on the 8 subsets together (4 positive subsets and 4 negative subsets), and then applied to the remaining subset as the test data set. These procedures were repeated 5 times for each test data set. In the ROC analysis, compounds reported to have potential cardiotoxicity in the literature were deemed positive regardless of the presence or absence of histopathological changes in the hearts under this study condition. Individual ΔΔCt and plasma cTnI values were used to construct the ROC plot for the analysis of the gene expression data and the cTnI data, respectively. R software was used for the ROC analysis with the 5-fold cross validation and SVM modeling. The expression patterns of the genes used to construct the multi-gene biomarker were also evaluated using the principal component analysis (PCA).

Statistical analysis:

GraphPad Prism version 4.03 (GraphPad Software Inc., San Diego, CA, USA) was used for the statistical analyses. The cTnI data were compared using one-way ANOVA followed by the Dunnett’s multiple comparison test. p values less than 0.05
were considered statistically significant.
Results

Sequential changes in histopathology and gene expression profiles:

Histopathological changes, including degeneration or necrosis of myocardial cells, edema in the interstitium, and/or infiltration of inflammatory cells in the left ventricle and/or interventricular septum, were observed at 8 and 24 h after dosing in isoproterenol-, metaproterenol-, and allylamine-treated animals (Figure 1A, 1C, 1D; Table 2). Although doxorubicin treatment resulted in edema in the interstitium in one animal at 24 h after dosing, there was no clear evidence of degenerative change (Figure 1B, Table 2). The known cardiotoxic compounds, mitoxantrone, cyclophosphamide, cyclosporine A, and allyl alcohol, did not induce any histopathological lesions in the heart at either of the sampling points under this study condition (Table 2). In addition, none of the non-cardiotoxic compounds induced any histopathological lesions in the hearts at either of the sampling points.

Figure 2 shows the expression profiles for the 8 genes at 8 and 24 h after dosing. The optimal cutoff value for each gene was calculated by the ROC analysis. Almost all genes were up-regulated at both 8 and 24 h after dosing in animals with histopathological cardiotoxic lesions. Additionally, some genes showed up-regulation in the animals treated with cardiotoxic compounds even though there was no cardiac lesion. Although these 8 genes were selected as candidate biomarkers based on the analysis in the chapter 1, there were many false positive predictions in some genes, such as observed for Lmcd1 and Tgfb2.
### Table 2. Histopathological findings in the heart following single treatment with cardiotoxic compounds

<table>
<thead>
<tr>
<th>Sampling point</th>
<th>Treatment</th>
<th>Findings</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 h</td>
<td>Isoproterenol</td>
<td>N (++<del>+++), I (+</del>++)</td>
<td>LV, S</td>
</tr>
<tr>
<td></td>
<td>Metaproterenol</td>
<td>N (+++)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Metaproterenol</td>
<td>I (+~++)</td>
<td>LV, S</td>
</tr>
<tr>
<td></td>
<td>Metaproterenol</td>
<td>E (+<del>+++), I (+</del>++)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mitoxantrone</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Allylamine</td>
<td>D (+, 1/3*)</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>Allyl alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>Isoproterenol</td>
<td>N (+++)</td>
<td>LV, S</td>
</tr>
<tr>
<td></td>
<td>Metaproterenol</td>
<td>N (+<del>++++), I (+</del>++)</td>
<td>LV, S</td>
</tr>
<tr>
<td></td>
<td>Metaproterenol</td>
<td>E (+<del>++), I (+</del>++)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mitoxantrone</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Allylamine</td>
<td>N (+, 2/3*), I (+, 2/3*)</td>
<td>LV, S</td>
</tr>
<tr>
<td></td>
<td>Allyl alcohol</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cyclosporine A</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Allyl alcohol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Findings**
- N: necrosis of myocardium
- I: infiltration of inflammatory cells
- E: edema of interstitium
- D: degeneration of myocardium
- ±: very slight
- #: slight
- ++: moderate
- +++: severe
- −: no change
- *: incidence

**Grade**
- LV: left ventricle
- S: septum
Sequential changes of plasma cTnI:

An increase in the plasma cTnI level was observed in isoproterenol-, metaproterenol-, and allylamine-treated groups in Exp. 1 (Figure 3). The elevation of the plasma cTnI tended to be transient, with detection mainly noted at early sampling points such as at 2 and 4 h after dosing. There were no changes in the level of the plasma cTnI in animals administered with the other cardiotoxic compounds or with any non-cardiotoxic compounds.

Verification of candidate genomic biomarkers and construction of a multi-gene biomarker:

To assess and compare the diagnostic accuracy of the 8 candidate genomic biomarkers, the author examined the gene expression data by a ROC analysis with a 5-fold cross validation using the data from Exp.1 and Exp.2. The analysis was conducted separately in accordance with each of the sampling points. ROC curves were plotted on the basis of the set of sensitivity and 1 − specificity (false positive rate) values (Figures 4A, 4B). The area under the ROC curve (AUC) values for the 8 candidate biomarker genes ranged from 0.092 (Tgfb2) to 0.866 (Timp1) at 8 h after dosing, and from 0.035 (Fhl1) to 0.906 (Spp1) at 24 h after dosing (Table 3). To achieve the best predictive performance, the author constructed an optimized multi-gene model using SVM. The genes with AUC values greater than 0.8 in the ROC analysis were selected for the multi-gene model at each sampling point: Timp1, Rnd1, Bcat1, and Fhl1 were selected for the 8 h point; and Spp1 and Timp1 were selected for the 24 h point. The
diagnostic accuracy of the newly constructed multi-gene model for each sampling point was compared by the ROC analysis with a 5-fold cross validation. The multi-gene model for 24 h after dosing was found to be superior to the model for 8 h, with the AUC values of the model for 8 h and 24 h after dosing determined to be 0.828 and 0.886, respectively (Table 3, Figure 4C). Based on these results, the 24 h model consisting of Spp1 and Timp1 was selected as the most optimized model and subsequently used in all further analyses.

<Constructed multi-gene biomarker>

\[ (A-0.794) ÷ 2 \times 0.731 + (B-0.547) ÷ 1.05 \times 0.507-1.04 \]

A: Spp1 \(\Delta\Delta\text{Ct}\),  B: Timp1 \(\Delta\Delta\text{Ct}\)

Cutoff value = −0.77

The calculation formula and cutoff value were calculated by R software.

<table>
<thead>
<tr>
<th></th>
<th>AUC at 8h</th>
<th>AUC at 24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Timp1)</td>
<td>0.866</td>
<td>0.906</td>
</tr>
<tr>
<td>(Rnd1)</td>
<td>0.845</td>
<td>0.843</td>
</tr>
<tr>
<td>(Bcat1)</td>
<td>0.827</td>
<td>0.773</td>
</tr>
<tr>
<td>(Fhl1)</td>
<td>0.812</td>
<td>0.667</td>
</tr>
<tr>
<td>(Serpine1)</td>
<td>0.601</td>
<td>0.634</td>
</tr>
<tr>
<td>(Spp1)</td>
<td>0.410</td>
<td>0.402</td>
</tr>
<tr>
<td>(Lmcd1)</td>
<td>0.124</td>
<td>0.279</td>
</tr>
<tr>
<td>(Tgfb2)</td>
<td>0.092</td>
<td>0.035</td>
</tr>
<tr>
<td>Multi-gene</td>
<td>0.828</td>
<td>0.886</td>
</tr>
</tbody>
</table>
Comparison of diagnostic accuracy between the multi-gene biomarker and the classical methods:

Data from Exp. 1 was used to compare the diagnostic accuracy of the newly constructed multi-gene biomarker consisting of Spp1 and Timp1 and the plasma cTnI measurement by the ROC analysis. The AUC value of the multi-gene biomarker was higher than the AUC values for the plasma cTnI measurement at all of the sampling points (Figure 4D, Table 4). The results confirmed that the multi-gene biomarker was superior to the plasma cTnI measurement.

Table 4. Comparison between a multi-gene biomarker and cTnI by ROC analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multi-gene</td>
<td>0.912</td>
</tr>
<tr>
<td>cTnI (2 h)</td>
<td>0.806</td>
</tr>
<tr>
<td>cTnI (4 h)</td>
<td>0.657</td>
</tr>
<tr>
<td>cTnI (8 h)</td>
<td>0.514</td>
</tr>
<tr>
<td>cTnI (24 h)</td>
<td>0.778</td>
</tr>
<tr>
<td>cTnI (all timepoints)</td>
<td>0.709</td>
</tr>
</tbody>
</table>

Early predictive performance of the multi-gene biomarker:

To confirm whether or not the multi-gene biomarker consisting of Spp1 and Timp1 would be applicable for the early and sensitive detection of cardiotoxicity in rats, the predictive performance of the multi-gene biomarker was confirmed in animals administered with doxorubicin at a lower dose level (3 mg/kg/day) for 1 or 7 days. Neither histopathological changes in the heart nor elevations in the plasma cTnI levels were found for any of the sampling points after 1- and 7-day dosing. The multi-gene
biomarker judged the gene expression data after 7-day dosing to be cardiotoxicity-positive, while the gene expression data after 1-day dosing was judged to be cardiotoxicity-negative (Figure 5).

Using PCA analysis, the author further examined the Spp1 and Timp1 gene expression data and determined that the values of rats dosed with doxorubicin for 1 day were plotted close to the cardiotoxicity-negative compounds. On the other hand, values for the rats administered with doxorubicin for 7 days exhibited a distinct separation from the cardiotoxicity-negative compounds, with the plot of the values toward the PC1 direction (89.2%) and close to the cardiotoxicity-positive compounds (Figure 6).
Discussion

In the chapter 2, the author used a large number of cardiotoxic and non-cardiotoxic compounds in order to examine the expression of 8 genes (*Spp1*, *Fhl1*, *Timp1*, *Serpine1*, *Bcat1*, *Lmcd1*, *Rnd1*, and *Tgfb2*) and to confirm their potentials as genomic biomarkers for cardiotoxicity in rats. After dosing of cardiotoxic compound, these 8 genes were commonly up-regulated at both 8 and 24 h in the hearts of rats. Many of these genes could detect the potential cardiotoxicity of the compound even in the absence of any distinct cardiac lesions. However, many false positive predictions were detected for some genes, and it was considered to be inappropriate to separately adopt all of these genes as biomarkers. In order to achieve higher diagnostic accuracy, these 8 genes were ranked according to the AUC values of the ROC analysis. Based on these results, an optimal multi-gene biomarker consisting of *Spp1* and *Timp1* were newly constructed using gene expression data at 24 h after dosing. The AUC value of the multi-gene biomarker (0.886) was comparable to that observed for *Spp1* (0.906), which had the highest performance in the ROC analysis. However, the multi-gene biomarker was considered to be more useful than when using only *Spp1* because of the concomitant application of *Timp1*. *Timp1* exhibited a high diagnostic accuracy sequentially at both 8 and 24 h after dosing, and the concomitant application of such a sequentially up-regulated gene should increase the diagnostic accuracy in preclinical studies, a phase during which various compounds with differing toxicokinetics profiles and toxicity mechanisms are usually evaluated. Although *Spp1* and *Timp1* showed high sensitivity at 24 h after dosing, there were false positive predictions in some compounds such as α-naphthylisothiocyanate and chloroform. These compounds are not familiar as a
cardiotoxic compound. However, it was reported that chloroform has a toxic effect on isolated rat cardiomyocytes (El-Shenawy and Abdel-Rahman, 1993) and that α-naphthylisothiocyanate-induced cholestasis might have an effect on heart mitochondria in rats (Oliveira et al, 2003). From these reports, the positive responses of Spp1 and Timp1 observed in this chapter might be related with the minute cardiotoxicity caused by these compounds. In this chapter, the author used verapamil and aconitine, compounds causing electrocardiographic alterations. Since Spp1 and Timp1 were not up-regulated by these compounds, it was suggested that these genes might not be up-regulated via electrocardiographic alterations. Thus, the multi-gene biomarker might be able to distinguish between degenerative structural and electrophysiological effects.

Considering an appropriate biomarker to be used in preclinical studies, biological interpretation of selected genes is important. The involvement of Spp1 in the development of cardiotoxicity has been widely reported. For example, osteopontin, the protein product of Spp1, promotes cardiac fibrosis via the differentiation of fibroblasts into myofibroblasts (Lenga et al., 2008; Zahradka, 2008). Although Spp1 is not expressed in the un-stressed heart, its expression has been shown to increase in animal models under pathological conditions such as cardiac infarction (Komatsubara et al., 2003). Expression of SPP1 and its protein product also increases in human dilated cardiomyopathy (Satoh et al., 2005; Stawowy et al., 2002). Timp1 is also a well-known cardiotoxicity-related gene. Timp1 plays a role in fibrosis during cardiac remodeling in mice and has been shown to be up-regulated during heart failure in humans (Barton et al., 2003; Szalay et al., 2009). Other studies have indicated that the balance between TIMP and matrix metalloproteinase (MMP) influences the development of cardiotoxicity in both rats and humans (Heymans et al., 2005; Peterson et al., 2000). As described above,
$Spp1$ and $Timp1$ are biologically relevant genes for the detection of cardiotoxicity in preclinical studies.

Both $Spp1$ and $Timp1$ have been shown to be functionally involved in ECM synthesis. It has been reported that ECM plays a central role in the remodeling processes after cardiac injury (Graham et al., 2008; Spinale, 2002). However, it should be noted that these genes demonstrated a high diagnostic performance even in rats treated with the cardiotoxic compounds, mitoxantrone, cyclophosphamide, cyclosporine A, and allyl alcohol, all of which did not induce any histopathological cardiotoxic lesions. Furthermore, after administration of 3 mg/kg doxorubicin in rats for 7 days, the multi-gene biomarker was able to detect potential cardiotoxicity even though there were no histopathological lesions found in the hearts. It has been reported that a balance between ECM synthesis and degradation is maintained in the normal heart, and that this balance plays an important role in maintaining the geometrical structure and function of the heart (Caulfield and Borg, 1979). Moreover, ECM has been shown to be involved in maintaining tissue formation and homeostasis (Lukashev and Werb, 1998). Under repeated dosing conditions, cyclosporine A and mitoxantrone reportedly have induced histopathological lesions in the heart (Herman et al., 1997; Selkoki et al., 2007). Cyclophosphamide and allyl alcohol are known to have potential cardiotoxicity (Senthilkumar et al., 2006, Sklar et al., 1991; Toennes et al., 2002). Doxorubicin has been used to create a chronic cardiotoxicity animal model. It was reported that the repeated dosing of doxorubicin at 1.25 mg/kg, 4 times/week for 4 weeks or at 1 to 2 mg/kg/week for 10 to 14 weeks caused distinct cardiac lesions histopathologically (Kelishomi et al., 2008; Mettler et al., 1977). The dosing condition of doxorubicin in this chapter, 3 mg/kg/day for 7 days, was considered to be sufficient to cause the
cardiotoxicity in an early phase without histopathological lesions, since the dose level was higher than those generally used to constructing a chronic cardiotoxicity model in rats using the compound. Overall, it was considered that a 7-day administration of doxorubicin at a low dose level or the single administration of other cardiotoxic compounds in this chapter might be able to cause a loss of cardiomyocyte homeostasis along with an imbalance of the ECM synthesis/degradation. Since in general pharmaceutical companies evaluate a wide variety of drug candidates, experimental tools that can be applicable to all drug classes would be of tremendous benefit for the drug development process. Considering these situations, the multi-gene biomarker would be very helpful for preclinical studies, since the loss of homeostasis in the myocardium and subsequent imbalance of the synthesis/degradation of the ECM might be common hallmarks of the cardiotoxicity induced by various classes of toxicants.

In this chapter, the author confirmed that the diagnostic accuracy of the multi-gene biomarker consisting of \textit{Spp1} and \textit{Timp1} was superior to that of the plasma cTnI measurements by the ROC analysis under a single dosing condition. In addition, the multi-gene biomarker was able to predict the potential cardiotoxicity of doxorubicin under a repeated dosing condition at an early time point at which neither the elevation of plasma cTnI nor the histopathological changes were yet present. Thus, it appears that high diagnostic accuracy and predictive performance over classical tools are advantages of the multi-gene biomarker. The use of the multi-gene biomarker seems to be appropriate during the early phase of drug development, in which potential cardiotoxicity of drug candidates needs to be predicted even in the absence of any clear cardiac lesions. On the other hand, other recent studies have extensively reviewed the usefulness of cTn for preclinical application (O'Brien, 2008; Reagan, 2010). In this
chapter, it was also demonstrated that the elevation of plasma cTnI were well-correlated with myocardial necrosis. These results are reasonable, as a previous study has shown that cTnI is a component of the myocardium and upon cardiotoxicity, it leaks into the plasma (Korff et al., 2006). The plasma cTn can be commonly applied to both preclinical and clinical studies and used as a translational biomarker (O'Brien, 2008; Reagan, 2010). As such, monitoring of plasma cTn in the later phases of preclinical studies or especially during the development of drug candidates having cardiotoxicity risks may be of great benefit and improve the overall safety of the developmental process.

Selecting and applying appropriate biomarkers in accordance with the development phase and toxicity characteristics of candidate drugs is essential in drug development.

The newly developed multi-gene biomarker should be incorporated into the safety assessment to increase the possibility of identifying cardiotoxicity potentials early on during medical drug development, as a part of an integrated approach. That may potentially assists in the development of safer drugs.
Summary

In the chapter 1, 33 genes were identified as candidate genomic biomarkers for cardiotoxicity in rats. In the chapter 2, the author focused on 8 of these 33 genes (\textit{Spp1}, \textit{Fhl1}, \textit{Timp1}, \textit{Serpine1}, \textit{Bcat1}, \textit{Lmcd1}, \textit{Rnd1}, and \textit{Tgfb2}) from their functions, and confirmed their usefulness using more cardiotoxic and non-cardiotoxic compounds. Additionally, an optimized support vector machine (SVM) model was constructed in order to achieve the best diagnostic performance. The 8 genes were ranked by a receiver operating characteristic (ROC) analysis, and the multi-gene model composed of \textit{Spp1} and \textit{Timp1} was newly constructed. This novel multi-gene biomarker exhibited a much higher diagnostic accuracy than that observed for plasma cardiac troponin I (cTnI), which is one of the most useful plasma biomarkers for cardiotoxicity detection. Furthermore, it was determined in a repeated dosing condition that the multi-gene biomarker could predict potential cardiotoxicity in rats prior to cardiac histopathological changes or elevations of plasma cTnI. Overall, the multi-gene biomarker exhibited advantages over classical tools owing to its high diagnostic accuracy and predictive performance. Therefore, it seems that an application of the multi-gene biomarker is suitable especially in the early phase of the drug development, in which any toxicity potentials of candidate drugs should be detected even when there is no apparent histopathological lesion. The application of the multi-gene biomarker could potentially lead to the production of safer drugs.
Figure 1. Histopathological changes in the myocardium. (A) The left ventricle of an isoproterenol-treated rat at 24 h after treatment. (B) The septum of a doxorubicin-treated rat at 24 h after treatment. (C) The left ventricle of a metaproterenol-treated rat at 8 h after treatment. (D) The left ventricle of an allylamine-treated rat at 24 h after treatment. (E) The left ventricle of control animal. Necrosis of the myocardium (arrows), interstitial edema (asterisks), and infiltration of inflammatory cells (arrowheads) were observed. Insets indicate a higher magnification of myocardial necrosis.
Figure 2. Gene expression profiles following treatment with cardiotoxic or non-cardiotoxic compounds. Real-time RT-PCR indicated that many of the genes were up-regulated at 8 h (A) and 24 h (B) after rats were treated with cardiotoxic compounds. Real-time RT-PCR: red indicates up-regulation (ΔΔCt=2) and blue indicates down-regulation (ΔΔCt=−2). *: group mean value was higher than cutoff value calculated by a ROC analysis.
Figure 3. Plasma cTnI level following treatment with cardiotoxic or non-cardiotoxic compounds at 2 h (A), 4 h (B), 8 h (C), and 24 h (D) after dosing. An elevation in the plasma cTnI level was observed at 2 h, 4 h, or 8 h after rats were treated with isoproterenol, metaproterenol, or allylamine. ANOVA-Dunnett’s: ** p < 0.01, * p < 0.05. The data represent the mean ± standard deviation.
Figure 4. ROC analysis of gene expression and plasma cTnI data. (A, B) A total of 8 genes were analyzed and prioritized by a ROC analysis at 8 h (A) and 24 h (B) after dosing of cardiotoxic or non-cardiotoxic compounds. (C) After constructing multi-gene models for each sampling point, the model for 24 h showed the higher diagnostic accuracy. (D) The multi-gene model for 24 h showed a higher diagnostic accuracy than the plasma cTnI measurement at any of the sampling points.
Figure 5. Predictive performance of the multi-gene biomarker in animals administered with doxorubicin at 3 mg/kg for 1 or 7 days. The multi-gene biomarker judged the gene expression data after 7-day dosing to be positive, while the gene expression data after 1-day dosing was judged to be negative. Cutoff value = −0.77.
Figure 6. The expression patterns of Spp1 and Timp1 by PCA. Plots of the data for rats treated with doxorubicin for 7 days were close to the data for the cardiotoxicity-positive compounds and toward the direction of PC1 (89.2%).
Chapter 3  Identification of a plasma miRNA biomarker for chemically-induced cardiotoxicity in rats
Introduction

In the chapter 2, the author constructed a multi-gene biomarker for sensitive detection of cardiotoxicity in rats for use in preclinical studies (Nishimura et al., 2013). The multi-gene biomarker showed a superior performance compared to plasma cardiac troponin I (cTnI), one of the conventional biomarkers for cardiotoxicity. However, application of the multi-gene biomarker requires sampling of the heart tissue, and such an assay cannot be applied to humans due to its invasive nature. In medical drug development, biomarkers which can be applied to both experimental animals and humans are ideal in order to monitor toxicity throughout a whole of developmental and post-marketing phases. Additionally, the risk-benefit balance is paramount in medication, and there are examples of therapies that use drugs with the cardiotoxic potential. For example, anti-cancer drugs such as anthracyclines, 5-fluorouracil, trastuzumab, and imatinib are known to induce cardiotoxicity (Broder et al., 2008). In the development of such drugs, a close monitoring of cardiotoxicity throughout preclinical and clinical studies has been essential. Plasma cTn (cTnI and cTnT) has been used as a useful translational biomarker in both preclinical and clinical studies (O’Brien, 2008; Reagan, 2010). However, plasma cTn shows transient elevation due to its rapid clearance, and the blood sampling time point therefore needs to be set appropriately for use in rats (Tonomura et al., 2012). Additionally, it has been reported that serum levels of cTn increase in patients with chronic kidney disease without cardiac symptoms (Ahmadi et al., 2014). These examples suggest potential limits to the usefulness of cTn; the development of novel translational biomarkers is still required.

MicroRNAs (miRNAs) are endogenous, small (~22-nucleotide) noncoding
RNAs. The existence of miRNAs in circulating blood has been reported, and some circulating (plasma/serum) miRNAs have been proposed as non-invasive, stable biomarkers for various pathological conditions (Haider et al., 2014). Recently, the relationships of miRNAs with cardiovascular diseases in humans have been reported (Oliveira-Carvalho et al., 2013), and the potential of circulating miRNAs as biomarkers have been shown in humans (van Empel et al., 2012). In experimental animals, the plasma levels of miR-1, miR-133a, miR-208a, and miR-499 were shown to be elevated in the rat acute myocardial infarction (AMI) model, with miR-208a appearing to be the most reliable among them (Wang et al., 2010). Plasma miR-208 was increased in isoproterenol-induced cardiotoxicity in rats (Ji et al., 2009). Nevertheless, limited data is available regarding circulating miRNA in experimental animals suffering cardiotoxicity, and the usefulness of plasma miRNAs as biomarkers for detecting cardiotoxicity in preclinical studies has not been adequately confirmed.

To be used as a plasma biomarker for tissue injury, the ideal miRNA should be abundantly and exclusively produced in the target organ, as large amounts of such a miRNA would then leak into circulating blood from the cytoplasm of the injured cells of the target organ. Recently, a miRNA dataset derived from as many as 55 different organs and tissues in normal rats has been reported (Minami et al., 2014). The dataset encompasses multiple relevant organs and tissues, and therefore is expected to be of great use for the selection of candidate miRNA biomarkers for toxicity of specific organ.

In the chapter 3, the author analyzed this comprehensive miRNA microarray dataset in the expectation that a miRNA that is expressed exclusively and abundantly in the target organ might serve as a promising biomarker. The author selected miRNAs
that were expressed exclusively and abundantly in the heart or the skeletal muscle from the standpoint that cardiotoxicity should be distinct from skeletal muscle toxicity by using a biomarker. Next, the author confirmed the usefulness of the selected miRNAs as plasma biomarkers for cardiotoxicity in rats administered with isoproterenol or doxorubicin, well-known cardiotoxic compounds. The author further evaluated the usefulness of the selected miRNA as a plasma biomarker for cardiotoxicity caused by a repeated dose with isoproterenol, since biomarkers for preclinical studies ideally should be applicable for chronic pathological conditions under a repeated dosing condition.
Materials and Methods

Chemical compounds:

Isoproterenol hydrochloride (Sigma-Aldrich, St Louis, MO, USA) and doxorubicin hydrochloride (Kemprotech Ltd., Middlesbrough, UK) were used. Hereinafter, these compounds are referred to by their free form names, and the dose levels are referred to as their free forms.

Animals and housing conditions:

Male Sprague-Dawley rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The animals were housed under a controlled temperature (20 – 26°C), humidity (30 – 70%), and light cycle (lights from 8 a.m. until 8 p.m.). All animals were allowed free access to food (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and water (sterilized city water via an automatic watering system with watering nozzles). The animals were 6 weeks of age at the initial dosing.

Animal experimental design:

All experimental procedures were conducted after the approval by the Institutional Animal Care and Use Committee.

Experiment (Exp.) 1 was conducted in order to confirm the time course profile of plasma miRNAs under a single dosing condition. Animals (n=4–5/group) were
administered with isoproterenol (1 mg/kg) or doxorubicin (30 mg/kg) as a single intravenous dose via the tail vein. Control animals were treated by the same route with equivalent volumes of physiological saline. A dose level previously shown to cause histopathological changes in the heart was selected for isoproterenol, and a maximally tolerable dose level (as determined in pilot studies) was selected for doxorubicin. After dosing, blood samples (0.3 mL at each time point) were collected via the tail vein at 2, 4, 8, and 24 h after dosing; and aliquots (0.2 mL for miRNA assay, 0.1 mL for troponin assay) were used for the measurement of miRNAs, cTnI, cTnT, and skeletal TnI (sTnI). K$_2$-EDTA was used as the anticoagulant for miRNA measurement; Na heparin was used as the anticoagulant for the measurement of cTnI, cTnT, and sTnI. Following collection, samples were separated by centrifugation at 1500×g for 15 min under refrigeration, and the resulting plasma specimens were stored frozen at −80°C pending analysis. After the final blood collection at 24 h after dosing, all animals were euthanized by cutting both the abdominal aorta and vena cava under pentobarbital sodium anesthesia. The heart and skeletal muscle (gastrocnemial and soleus) then were collected for histopathological examination.

Exp. 2 was conducted in order to confirm the plasma miRNA profile in rats under a repeated dosing condition. Additionally, miRNA expression in the heart tissue was analyzed under this regimen. Isoproterenol was used in Exp. 2 because this compound caused distinct cardiac injury after a single dose in Exp. 1, and it was considered that the pathological condition in the heart would proceed to a sub-chronic phase such as fibrosis with repeated dosing. Animals (n=5/group) were administered with isoproterenol (0.5 mg/kg, intravenously via the tail vein) once daily for 7 days (Days 1–7), and blood samples (0.2 mL at each time point) were collected via the tail
ven for miRNA measurement on Days 2, 4, and 8. Each blood sampling was conducted approximately 24 h after administration of the previous day’s dose and prior to the administration of the next dose. The procedure for blood processing was the same as that used in Exp. 1. After the final blood collection on Day 8, all animals were euthanized and necropsied in the same manner as in Exp. 1, and the heart samples were collected for miRNA measurement and histopathological examination. Approximately 90 mg of heart tissue (obtained from the heart, and extending from the cardiac apex to the central portion of the left ventricle) was used for RNA extraction, and the remaining portion of the heart was used for histopathological examination. Other animals (n=5/group) were administered with single-dose isoproterenol at the same dose level, and the heart samples were obtained on Day 2 (approximately 24 h after dosing) for miRNA measurement and histopathology by the same process as above. Control animals (treated according to the same regimen, but with physiological saline) were included for each experimental condition.

RNA extraction:

For the analysis of plasma, frozen plasma specimens (100 µL at each point) were thawed and treated with QIAzol Lysis Reagent (Qiagen, Valencia, CA, USA), and total RNA then was extracted using a miRNeasy® Mini Kit (Qiagen) according to the manufacturer's protocol. For the analysis of heart tissue, frozen tissues were thawed and homogenized in QIAzol Lysis Reagent (Qiagen) using a TissueLyser (Qiagen), and total RNA then was extracted in the same manner as for plasma. The RNA concentration of the heart tissue-derived samples was determined using a Multiskan GO
Selection of miRNA based on microarray dataset:

MicroRNA expression profiles in 55 organs and tissues of normal rats were downloaded from the published data. The expression profiles were obtained using 8 x 15k customized Agilent Rat miRNA microarray containing both miRBase 15.0 and 16.0 probes, and all signal intensities were scaled to the 75th percentile of the median of the dynamic target value for each array using the central tendency normalization method (Minami et al., 2014, Gene Expression Omnibus GSE52754, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52754). Profiles comparing two different sampling conditions, with or without perfusion, were provided for a subset of four organs (heart, kidney, liver, and lung); the data from the perfused organs were used for the present analysis. The candidate miRNA biomarkers for cardiotoxicity or skeletal muscle toxicity were selected based on the expectation that miRNAs that are abundantly and exclusively produced in the target organ should quantitatively leak into the circulation after tissue injury.

First, miRNAs that were exclusively produced in the heart were selected. Base-2 logarithmic transformation was applied to miRNA expression levels in each organ, and the mean and standard deviation expression levels in all organs were calculated for each miRNA. Then, any miRNA whose expression was more than 2 standard deviations from the mean exclusively in the cardiac atrium and intraventricular septum (all tissues from the heart) was selected. From the selected miRNAs, miRNA that was abundantly produced in the heart was selected. In the analysis, the ratio of
miRNA expression level in each organ to the sum of expression level in all organs was calculated for each miRNA. Then, any miRNA whose expression ratio was more than 10% each in the cardiac atrium and intraventricular septum was selected.

Candidate miRNAs were additionally selected by milder criteria. miRNAs whose expressions were more than 1.5 standard deviations from the mean with expression ratio over 5% in the cardiac atrium and intraventricular septum were selected. The milder criteria did not yield additional miRNAs that met the criteria exclusively in heart tissue. Therefore, the author selected miRNAs that met the above criteria both in the heart (cardiac atrium and intraventricular septum) and skeletal muscle (femoris muscle, gastrocnemial muscle, and soleus muscle). In this selection, the expression exceeding the criteria in the other muscular tissues (interseptum and tongue) was accepted.

Next, the author selected skeletal muscle-specific miRNAs in order to distinguish cardiotoxicity from skeletal muscle toxicity. In this selection, miRNAs whose expressions were more than 2 standard deviations from the mean with expression ratio over 10% exclusively in the femoris muscle, gastrocnemial muscle, and soleus muscle were selected. In this selection, the expression exceeding the criteria in the other muscular tissues except the heart (interseptum and tongue) was accepted.

Quantification of miRNA by real-time RT-PCR:

The plasma levels of miR-1, miR-133a, miR-133b, miR-206, and miR-208 were measured using a quantitative real-time RT-PCR assay. The assay used TaqMan® MicroRNA Reverse Transcription Kit and TaqMan® miRNA Assays
(Applied Biosystems, Foster City, CA, USA), employing the following kits (selected based on miRBase Accession Number): rno-miR-1, assay ID 002064; hsa-miR-133a, assay ID 002246; hsa-miR-133b, assay ID 002247; hsa-miR-206, 000510; and rno-miR-208, assay ID 463567_mat. Following reverse transcription reaction, cDNA (2 µL/rat/sampling point) was amplified using TaqMan® Universal Master Mix, No AmpErase® UNG (Applied Biosystems), and each miRNA-specific primer included in TaqMan® miRNA Assays (Applied Biosystems). The RT-PCR procedure was conducted in accordance with the manufacturer’s protocols. Reverse transcription and amplification conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The resulting cycle threshold (Ct) value was determined based on the comparative Ct method. U6 small nuclear RNA (U6 snRNA, assay ID 001973) was used as an internal control.

Plasma troponin measurement:

Frozen plasma specimens were thawed and used for the measurement of cTnI, cTnT, and sTnI (skeletal muscle-specific biomarker) concentrations using an enzyme-linked immunoabsorbent assay kit (Meso Scale Discovery, Gaithersburg, MD, USA). The procedures were in accordance with the manufacturer’s protocols.

Histopathological examination:

The hearts and skeletal muscles (soleus and gastrocnemial) were fixed in 10% neutral buffered formalin, dehydrated in alcohol, and embedded in paraffin. After
paraffin sections were prepared and stained with hematoxylin and eosin (H&E) using standard methodologies, all samples were examined under a light microscope. In Exp. 2, the heart sections were additionally stained with Masson’s trichrome stain for the evaluation of fibrosis.

**Measurement of negative control plasma samples:**

In order to eliminate the possibility of false positive elevation of the selected miRNAs, the plasma miRNA expression in rats administered with non-cardiotoxic compound (negative controls) was confirmed. For the analysis, plasma samples obtained in another experiment were utilized (Tonomura et al., 2012). The non-cardiotoxic compounds were aminoglutethimide (Bachem Bioscience, Inc., PA, USA; 200 mg/kg, by oral gavage (po)), acetaminophen (Sigma-Aldrich; 1000 mg/kg, po), and methapyrilene hydrochloride (Sigma-Aldrich; 500 mg/kg, po). Aminoglutethimide is known to cause toxicity in the adrenal gland, while acetaminophen and methapyrilene are known to cause toxicity in the liver. In these control experiments, rats (n=3/group) were administered with a single dose of the respective compound, and K₂-EDTA blood samples were obtained (under pentobarbital sodium anesthesia) from the abdominal vena cava at approximately 24 h after dosing. Samples were processed and stored (frozen at −80°C) pending use. No histopathological lesions were observed in the hearts and skeletal muscles of these animals (Tonomura et al., 2012).

**Statistical analysis:**
GraphPad Prism version 4.03 (GraphPad Software Inc., San Diego, CA, USA) was used for the statistical analyses of the data of miRNAs, cTnI, cTnT, and sTnI. ANOVA-Dunnett’s test was applied for comparisons among 3 groups, and two-tailed $t$-test with Welch's correction was applied for the comparison between 2 groups, respectively. p values less than 0.05 were considered statistically significant.
Results

Selection of candidate miRNAs from microarray data:

The selection process of candidate miRNAs is summarized in Figure 1. miRNAs whose expression levels were more than 2 standard deviations from the mean exclusively in the cardiac atrium and intraventricular septum were rno-miR-185, rno-miR-208, rno-miR-208_v15.0, rno-miR-22, rno-miR-30c, rno-miR-30d, and rno-miR-345-5p. Among these candidates, only rno-miR-208 and rno-miR-208_v15.0 separately showed expression ratios exceeding 10% in the cardiac atrium and intraventricular septum. The sum of the expression ratios in the cardiac atrium and interventricular septum achieved values exceeding 90% of the total expression in all organs. On the other hand, these miRNAs showed low expression levels (less than 1%) in all other organs, including the other muscular tissues. rno-miR-208_v15.0 was eliminated from consideration because the base sequence of the probe was not identical to that of the rat miR-208.

Further selection was conducted with milder criteria. miRNAs whose expression levels were more than 1.5 standard deviations from the mean in the heart (cardiac atrium and intraventricular septum) and skeletal muscle (femoris muscle, gastrocnemial muscle, and soleus muscle) were rno-miR-1, rno-miR-133a, rno-miR-133a*, and rno-miR-133b. All of these candidates showed expression ratios exceeding 5% in the above-specified muscle tissues. The expression ratio in the heart (16–20%, the sum of the expression ratios of cardiac atrium and intraventricular septum) was lower than that in the skeletal muscle (51–57%, the sum of the expression...
ratios of the femoris muscle, gastrocnemial muscle, and soleus muscle). There were also relatively high levels of expression in the other muscle tissues (19–21%, the sum of the expression ratios of the interseptum and tongue). On the other hand, extremely low expression levels (representing expression ratios of less than 1%) were observed in almost all other organs, with the exceptions being expression ratios of 2–4% and 1% in the esophagus and skin, respectively.

In the selection of the skeletal muscle-specific miRNAs, miRNAs whose expression levels were more than 2 standard deviations from the mean exclusively in the femoris muscle, gastrocnemial muscle, and soleus muscle were selected; only rno-miR-206 met these criteria. rno-miR-206 showed expression ratio exceeding 10% in each of three surveyed muscle tissues (femoris, gastrocnemial, and soleus), with the sum of the expression ratios across the three reaching as high as 68% of the total expression in all organs. Additionally, there was relatively high expression in the other muscular organs (21%, the sum of the expression ratios in the interseptum and tongue). On the other hand, rno-miR-206 showed extremely low expression in almost all other organs, including the heart (less than 1%). The exceptions were the expression ratios of 7% and 2% in the esophagus and skin, respectively.

From the above results, miRNAs were selected as candidate plasma miRNA biomarkers as follows: miR-208 (rno-miR-208a-3p) for cardiotoxicity; miR-1 (rno-miR-1-3p), miR-133a (rno-miR-133a-3p), miR-133a* (rno-miR-133a-5p), and miR-133b (rno-miR-133b-3p) for shared cardiotoxicity and skeletal muscle toxicity; and miR-206 (rno-miR-206-3p) for skeletal muscle toxicity. Expression patterns of each miRNA in a comprehensive set of organs and tissues are shown in Figure 2. miR-133a and miR-133a* showed similar expression patterns; in subsequent real-time
RT-PCR assays, only miR-133a was measured.

**Histopathological changes in the heart and skeletal muscle and sequential changes of plasma cTnI, cTnT, and sTnI after a single administration of isoproterenol or doxorubicin:**

Histopathological evaluation revealed the induction in the heart of distinct necrosis of myocardial cells, edema, and infiltration of inflammatory cells after a single dose of isoproterenol (Figure 3, Table 1). There were no changes in the skeletal muscle. On the other hand, no abnormal changes were induced in the heart or skeletal muscle after a single dose of doxorubicin (Table 1).

The plasma concentrations of cTnI and cTnT were elevated after a single dose of isoproterenol (Figure 4). Although the elevation remained significant until 24 h after dosing only for cTnI, the elevations of cTnI and cTnT concentrations were detected mainly at the early sampling points. There were no significant changes in the sTnI concentration compared to the control at any of the sampling points. In the rats administered with single dose doxorubicin, an elevation was observed only for the sTnI concentration at 24 h after dosing; no elevations were seen for the cTnI and cTnT concentrations at any of the sampling points (Figure 4).
Sequential plasma miRNA levels after a single dose of isoproterenol or doxorubicin:

The plasma levels of miR-1, miR-133a, miR-133b, miR-206, and miR-208 were measured sequentially in rats administered with single-dose isoproterenol or doxorubicin (Figure 5). Among the aforementioned miRNAs, plasma miR-208 was sustainably increased from the first sampling point through 24 h after dosing with isoproterenol. Plasma miR-133b showed an increase only at 8 h after dosing. There were no significant changes in the levels of plasma miR-1, miR-133a, and miR-206 after a single dose of isoproterenol. In rats administered with doxorubicin, plasma miR-206 was increased at 8 and 24 h after dosing; miR-1, miR-133a, and miR-133b were increased only at 24 h after dosing. On the other hand, there was no significant change in the plasma miR-208 level after dosing with doxorubicin.
Specificity of selected plasma miRNAs in negative control:

There were no significant changes in any of the tested miRNAs in plasma of rats administered with each of the non-cardiotoxic compounds (aminogluthethimide, acetaminophen, and methapyrilene) (Figure 6).

miR-208 level in plasma and heart tissue after 7-day repeated dosing of isoproterenol:

In histopathology (Figure 3, Table 2), fibrosis was observed primarily in the heart after 7-day dosing with isoproterenol. On the other hand, necrosis of cardiomyocytes and infiltration of inflammatory cells were diminished upon repeated dosing. Plasma miR-208 was sustainably increased on Day 2, Day 4, and Day 8 (though the change on Day 4 was not statistically significant) (Figure 7). On the other hand, the expression of miR-208 in heart tissue was not upregulated under either a single or repeat dose condition (Figure 7).
Table 2. Histopathological findings in the heart following a single or 7-day repeated treatment with isoproterenol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1-day</th>
<th>7-day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoproterenol</td>
<td>N (LV, S): +~++</td>
<td>N (LV, S): −~+</td>
</tr>
<tr>
<td></td>
<td>E (LV, S): −~++</td>
<td>I (LV, S): −~++</td>
</tr>
<tr>
<td></td>
<td>I (LV, S): +~++</td>
<td>F (LV, S): +~+++</td>
</tr>
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<table>
<thead>
<tr>
<th>Findings</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>N: necrosis of myocardium</td>
<td>+: slight</td>
</tr>
<tr>
<td>I: infiltration of inflammatory cells</td>
<td>++: moderate</td>
</tr>
<tr>
<td>E: edema of interstitium</td>
<td>+++: severe</td>
</tr>
<tr>
<td>F: fibrosis</td>
<td></td>
</tr>
<tr>
<td>−: no change</td>
<td></td>
</tr>
</tbody>
</table>

Site
LV: left ventricle
S: septum
Discussion

In the chapter 3, the author selected miRNAs as candidate plasma biomarkers for cardiotoxicity, and confirmed their usefulness using cardiotoxic compounds under both single and repeated dosing conditions. In an initial screening, the author analyzed a miRNA microarray dataset across a comprehensive range of rat organs and tissues (Minami et al., 2014). Based on this initial analysis, miR-208 was selected as the first candidate due to its exclusive and abundant expression in the heart. Additionally, miR-1, miR-133a, and miR-133b were selected as the secondary candidates. Although these 3 miRNAs showed relatively exclusive and abundant expression in the heart, the expression levels were higher in the skeletal muscle than in the heart, and it appears that these 3 miRNAs would be more appropriate as biomarkers for skeletal muscle toxicity. In medical drug development, the target organ for toxicity should be distinguished by organ-specific biomarkers. Therefore, the author selected miR-206 as the skeletal muscle-specific miRNA in order to distinguish cardiotoxicity from skeletal muscle toxicity for use in combination with miR-1, miR-133a, and miR-133b.

The author confirmed the usefulness of the above candidate miRNAs using a rat model of drug-induced cardiotoxicity. After a single dose of isoproterenol, plasma miR-208 sustainably showed an increase accompanied by histopathological changes in the heart and elevations of plasma cTnI and cTnT concentrations. As for the other miRNAs, only miR-133b showed a transient increase; no significant changes were observed in the plasma levels of miR-1, miR-133a, and miR-206. Such sensitive elevation of the plasma miR-208 level seems to be owing to this molecule’s exclusive and abundant expression in the heart tissue compared to miR-1, miR-133a, and miR-133b.
These data suggest that plasma miR-208 is a promising biomarker for cardiotoxicity in rats. Furthermore, the author confirmed that the increased plasma miR-208 persisted at similar levels even after 7 days of once-daily dosing of isoproterenol, although histopathological lesions changed to the sub-chronic phase of fibrosis with diminished necrotic lesions during the course of this repeat-dose regimen. Such sustained elevation of the plasma miR-208 level under the repeated dosing conditions is ideal for a biomarker for use in preclinical studies, since cardiotoxicity usually is evaluated and detected under the repeated dosing conditions.

The author compared the plasma profile of miR-208 to that of cTn after single-dose isoproterenol. Interestingly, the time course profile of plasma miR-208 was different from those of cTnI and cTnT: the level of miR-208 was sustained through 24 h after dosing, while the plasma concentrations of cTnI and cTnT almost returned back to the control levels by 24 h after dosing. In preclinical studies, drug candidates with various toxicity mechanisms and pharmacokinetic profiles are evaluated, and the selection of appropriate blood sampling time points is required for the measurement of plasma cTn due to its transient elevation. Therefore, the sustainable increase of plasma miR-208 is expected to lead to easier detection of cardiotoxicity in medical drug development. Under the conditions of the present study, the expression of miR-208 in the heart did not differ significantly under both single and repeated dosing conditions. The sustainable elevation of plasma miR-208 level might be due to slow elimination from circulation. Mature miRNAs are incorporated into RNA-induced silencing complexes (RISCs) in the cytoplasm of mammalian cells (Bartel, 2004). It has been reported that the major portion of circulating miRNAs in humans is bound to Argonaute 2, one of the essential components of RISC (Arroyo et al., 2011; Wang et al., 2012). The molecular
weight of Argonaute is approximately 100 kDa in mice and humans (Wang et al., 2012). It has been reported that miR-208 released from the heart was detected in the urine of the rat AMI model (Cheng et al., 2012). Based on these reports, the sustainable elevation of plasma miR-208 observed in this chapter might reflect low renal clearance attributable to binding by RISC proteins. While it is assumed that plasma miR-208 is eliminated mainly via the kidneys, plasma miR-208 levels could be influenced by the glomerular filtration rate, as seen in the similar case of plasma troponins (Ahmadi et al., 2014). Interestingly, the plasma miR-208 level was not changed in rats with renal infarction (Ji et al., 2009).

On the other hand, the levels of plasma miR-1, miR-133a, miR-133b, and miR-206 were elevated after single-dose doxorubicin. These miRNA changes presumably were caused by skeletal muscle injury, since there was concomitant elevation of plasma concentrations of sTnI, an established biomarker for skeletal muscle injury (Tonomura et al., 2012). The increase of plasma miRNAs was observed at later sampling points such as 8 h and/or 24 h after dosing. The time course profiles of these miRNAs were consistent with the profile of sTnI. It is possible that muscular toxicity caused by doxorubicin occurs at a later time point, since gene expression in the heart was more prominent 24 h after dosing with doxorubicin compared to 8 h in the chapter 1 (Mori et al., 2010). There was no elevation of plasma miR-208 level after dosing with doxorubicin, contrary to the author’s expectation. In this chapter, there were no histopathological changes in the heart or elevation in the plasma concentrations of cTnI and cTnT induced by doxorubicin. Apparently, cardiotoxicity was not induced in rats administered with doxorubicin under the condition of the present study.

Recently, the potential for circulating miR-208 as a biomarker for
cardiovascular disease in humans has been shown (Li et al., 2013; van Empel et al., 2012; Wang et al., 2010). miR-208 (miR-208a-3p) is encoded by intron of MYH6 (myosin, heavy chain 6, cardiac muscle, alpha) in both humans (Location, chr14: 23857805-23857875) and rats (Location, chr15: 33051957-33052039) (Ensembl Genome Browser, http://asia.ensembl.org/index.html for humans, http://oct2012.archive.ensembl.org/index.html for rats), and this miRNA's sequence is highly conserved between the two species. Additionally, human miR-208 has been reported to be exclusively expressed in the heart (Wang et al., 2010). Considering these facts and the data obtained in this chapter, plasma miR-208 is a promising biomarker for cardiotoxicity, both in preclinical and clinical studies. It was a great satisfaction that the data of this chapter are consistent with previous reports suggesting the usefulness of miR-208 as a marker of cardiac injury in rats (Wang et al., 2010; Ji et al., 2009). The data increases the credibility of miR-208 as a plasma biomarker for cardiotoxicity in rats, since the author selected miR-208 using a comprehensive microarray dataset. Furthermore, the stable increase of plasma miR-208 was confirmed under the sub-chronic pathological conditions such as fibrosis, suggesting that sustained elevation was not due to upregulation in the heart tissue. Additionally, the author confirmed the specificity of all plasma miRNAs, including miR-208, using negative controls. The inclusion of data for negative controls is especially important for medical drug development, because false positive reactions of a biomarker could lead to an unnecessary cessation of the development of promising drug candidates.

In the chapter 3, it was concluded that plasma miR-208 appears to be a promising biomarker for drug-induced cardiotoxicity in rats. Recently, several studies have shown the usefulness of circulating miR-208 as a biomarker for cardiac injury in
humans. Although further studies are needed in humans, miR-208 might serve as a translational biomarker that could replace plasma cTn in medical drug development.
Summary

In medical drug development, biomarkers which can be applied to both experimental animals and humans are ideal in order to monitor toxicity throughout a whole of developmental and post-marketing phases. In the chapter 3, the author aimed to explore a plasma miRNA biomarker for cardiotoxicity in rats. Since organ-specificity is an important factor for a biomarker, the author analyzed the miRNA microarray dataset in 55 organs/tissues in normal male rats. Based on this analysis, 5 miRNAs consisting of miR-208 (heart-specific), miR-1, miR-133a, miR-133b (heart and skeletal muscle-specific), and miR-206 (skeletal muscle-specific) were selected. Next, the usefulness of those 5 miRNAs was evaluated using a rat model of single dose of isoproterenol or doxorubicin. Plasma miR-208 was consistently increased through 24 h after dosing in rats administered with isoproterenol, while plasma concentrations of cardiac troponin (cTn) showed transient elevation. On the other hand, the plasma levels of miR-1, miR-133a, miR-133a, and miR-206 were elevated following treatment with doxorubicin, probably due to skeletal muscle toxicity. Additionally, the plasma miR-208 level was elevated even after repeat-dose (once daily for 7 days) of isoproterenol under which the pathological condition proceeded to a sub-chronic phase such as fibrosis. Thus, it was suggested that miR-208 is a promising plasma biomarker for cardiotoxicity in rats. Monitoring of plasma miR-208 levels in rats may lead to more accurate evaluation of cardiotoxicity in preclinical studies.
Figure 1. Selection flow of candidate miRNAs. CA: cardiac atrium, IS: intraventricular septum, FM: femoris muscle, GM: gastrocnemial muscle, SM: soleus muscle. The selection was conducted from 2 standpoints; 1) miRNA expression was more than 2 (A, E) or 1.5 (C) standard deviations from the mean exclusively in each target organ (base-2 logarithmic transformation was applied), 2) miRNA expression ratio was more than 10% (B, F) or 5% (D) in each target organ. rno-miR-208_v15.0 was finally eliminated because the base sequence of the probe was not identical to that of the rat miR-208. In the selections C, D, E, and F, expression exceeding the criteria in the interseptum and tongue was accepted.
Figure 2. Expression profiles of selected miRNAs (miR-208, miR-1, miR-133a, miR-133a*, miR-133b, miR-206) in normal male rat organs/tissues. The ratio of each miRNA expression level in each organ to the sum of expression level in all organs was calculated. Each plot is arithmetic mean of the expression percentage of 3 animals.
Figure 3. Histopathological changes in the heart, as detected with hematoxylin and eosin (H&E) or Masson's trichrome staining. (A) The septum of a control rat in Exp.1 (H&E). (B) The septum of an isoproterenol (1 mg/kg) -treated rat at 24 h after treatment in Exp. 1 (H&E). (C) The septum of an isoproterenol-treated rat (0.5 mg/kg) on Day 2 in Exp. 2 (H&E). (D) The septum of an isoproterenol-treated rat (0.5 mg/kg) on Day 2
in Exp. 2 (Masson’s trichrome). (E) The septum of an isoproterenol-treated rat (0.5 mg/kg) on Day 8 in Exp. 2 (H&E). (F) The septum of an isoproterenol-treated rat (0.5 mg/kg) on Day 8 in Exp. 2 (Masson’s trichrome). Necrosis of the myocardium (arrows), intestinal edema (asterisks), and infiltration of inflammatory cells (arrowheads) were observed after single-dose isoproterenol (B, C, D). In contrast, fibrosis was mainly observed after 7 days of once-daily dosing with isoproterenol (E, F).

**Figure 4.** The sequential profile of plasma cTnI, cTnT, and sTnI levels following single treatment with isoproterenol (ISO) or doxorubicin (DXR). ANOVA-Dunnett’s: ** p < 0.01 (for ISO), * p < 0.05 (for ISO), ## p < 0.01 (for DXR). Each plot is mean ± standard deviation.
Figure 5. The sequential profile of plasma miRNAs following single treatment with isoproterenol (ISO) or doxorubicin (DXR). ANOVA-Dunnett’s test: ** p < 0.01 (for ISO), * p < 0.05 (for ISO), ## p < 0.01 (for DXR), # p < 0.05 (for DXR). Each plot is mean ± standard deviation.
Figure 6. Plasma miRNAs following treatment each of the non-cardiotoxic compounds. The data represent the mean ± standard deviation of each compound-treated group compared to control (n=3/group). There were no statistical significances by ANOVA-Dunnett’s test.

Figure 7. The sequential profile of miR-208 in plasma and heart tissue during 7-day repeat-dose treatment with isoproterenol. Two-tailed t-test with Welch's correction: ** p < 0.01, * p < 0.05. Each plot is mean ± standard deviation.
Conclusion

This study identified genomic biomarkers for chemically-induced cardiotoxicity in rats to be used in preclinical studies. The following results were obtained:

1. Three cardiotoxic compounds, isoproterenol, doxorubicin, or carbofuran were administered to rats a single time, and microarray analysis was conducted using heart tissues. As a result, it was revealed that 33 genes were commonly upregulated in accordance with cardiotoxicity induced by each of 3 different compounds. These genes were considered to be potential genomic biomarkers for cardiotoxicity in rats. (Chapter 1)

2. Eight genes (Spp1, Fhl1, Timp1, Serpine1, Bcat1, Lmcd1, Rnd1, and Tgfb2) were selected from the above 33 genes by their functions. The 8 genes were ranked by the ROC analysis using more cardiotoxic and non-cardiotoxic compounds, and an optimal multi-gene biomarker consisting of Spp1 and Timp1 was constructed. In the ROC analysis, the novel multi-gene biomarker showed higher diagnostic accuracy than cTnI in a single dosing condition. (Chapter 2)

3. The multi-gene biomarker detected cardiotoxicity induced by doxorubicin prior to histopathological changes or cTnI elevation in a repeated dosing condition. It was considered that the multi-gene biomarker could be a useful biomarker with high predictive performance. (Chapter 2)
4. From the analysis of a comprehensive miRNA dataset derived from 55 different organs/tissues in normal rats, miR-208 was selected as the candidate plasma biomarker for cardiotoxicity because of its exclusive and abundant expression in the heart. In cardiotoxicity caused by a single dose of isoproterenol, plasma miR-208 showed an elevation more sustainably in comparison with cTnI or cTnT. Such sustained elevation can lead to a sensitive detection of cardiotoxicity independently of blood sampling points. Additionally, plasma miR-208 showed a persistent elevation under a 7-day repeated dosing of isoproterenol, in which cardiotoxicity proceeded to a sub-chronic phase. (Chapter 3)

A series of the present studies explored two genomic biomarkers for chemically-induced cardiotoxicity in rats: the multi-gene biomarker using the heart tissue and plasma miR-208. The multi-gene biomarker showed higher diagnostic accuracy and predictive performance in comparison with histopathology or cTnI. Plasma miR-208 showed a sustained elevation in comparison with cTnI or cTnT. Therefore, the application of these novel biomarkers assists to prioritize candidate drugs more efficiently. As for plasma miR-208, non-invasiveness is an advantage. Several studies have shown the usefulness of circulating miR-208 as a biomarker for cardiac injury in humans. miR-208 might serve as a useful translational biomarker in the future.
References


Kaya, Z., Göser, S., Buss, S. J., Leuschner, F., Ottl, R., Li, J., Völkers, M., Zittrich, S.,


Oliveira-Carvalho, V., da Silva, M. M., Guimarães, G. V., Bacal, F. and Bocchi, E. A.
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Qureshi, Z. P., Seoane-Vazquez, E., Rodriguez-Monguio, R., Stevenson, K. B. and


Sheikh, F., Raskin, A., Chu, P. H., Lange, S., Domenighetti, A. A., Zheng, M., Liang, X.,


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