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Messenger ribonucleic acid expression profile in peripheral blood cells from RA patients following treatment with an anti-TNF-α monoclonal antibody, infliximab

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Objectives. We monitored the mRNA expression profiles of peripheral blood cells during treatment with a TNF-α inhibitor, infliximab, in patients with RA. Using a DNA microarray analysis, we demonstrated a unique set of genes, with distinct baseline and post-treatment changes in expression between responders and non-responders to infliximab treatment.

Methods. Using a customized low-density cDNA microarray with 747 genes and a reliable data collection system, we monitored the mRNA expression profiles of whole blood cells from 18 RA patients before and after the infusion of infliximab for up to 22 weeks. The clinical response to treatment with infliximab was determined using the ACR response criteria, the disease activity score of 28 joints (DAS28), and individual clinical parameters. The patients were classified as responders or non-responders based on their ACR50% response at 22 weeks.

Results. Approximately 15% of the total genes were found to exhibit a >1.5-fold change, compared with their reference values, at one or more time points during the 22 weeks of infliximab therapy. The expression of inflammatory genes, such as IFN-related genes, was strongly correlated with the serum level of CRP and the DAS28. The increased expression of inflammatory genes in responders was normalized within 2 weeks and then remained at a normal level during the treatment period. In contrast, in the non-responders, the elevated expression at baseline, although it was significantly decreased at 2 weeks, returned to the baseline level after 14 weeks. In addition to inflammatory genes, we identified several groups of genes with distinct differences in expression between the responders and non-responders.

Conclusions. Our results suggest that a customized low-density microarray is useful for monitoring mRNA expression profiles in peripheral blood cells, enabling us to identify a unique set of genes with differentially regulated expressions in responders and non-responders to a TNF inhibitor among patients with RA.

KEY WORDS: Rheumatoid arthritis, Oligonucleotide array sequence analysis, Biological products, Infliximab, Tumour necrosis factor, Interferons, Messenger ribonucleic acid, Reverse transcriptase-polymerase chain reaction.

Introduction

Biological agents that inhibit the action of TNF-α, such as infliximab, etanercept and adalimumab, have shown excellent clinical efficacy and a striking ability to prevent structural damage in patients with RA [1–4]. This clinical evidence supports the hypothesis that TNF-α lies upstream of the pro-inflammatory cytokine network and plays a pivotal role in the pathogenesis of RA [5]. Nevertheless, a satisfactory clinical response is not achieved in all patients treated with anti-TNF biologics. For example, the response to anti-TNF biologics in MTX-resistant RA patients is typically around 50–70% in terms of the ACR20% response criteria at 6 months [1–4]. To maximize the clinical response to these agents, the mechanism underlying the variable response in individual patients must be understood; furthermore, a strategy for predicting responders and non-responders is needed. Although clinical parameters, autoantibody profiles and biomarkers like serum MMP-3 have been studied [6–9], reliable prediction markers have not been identified to date. A pharmacogenomic analysis has also been performed [10]. Some studies have shown that single nucleotide polymorphisms (SNPs) in genes encoding TNF-α, TNF-α receptors, other cytokines and the MHC are significantly associated with a favourable response to ant-TNF biologics, while others conclude that such SNPs are irrelevant to predicting response [10–12]. In particular, a recent study in a large RA cohort demonstrated that a shared epitope was a marker of disease severity but was not a predictor of infliximab response [13]. Using a comprehensive analysis of mRNA expression profiles [14], synovial cells and peripheral blood cells from RA patients have been examined [15–17]. Based on the results of a transcriptome analysis, possible predictors of drug response are now being explored using not only synovial biopsy specimens, but also peripheral blood samples [18–20]. While baseline profiling is important for predicting a favourable response to a given drug, information regarding differences in the mRNA profiles of responders and non-responders is indispensable to understanding the molecular basis of drug response. To monitor the changes in mRNA expression following the administration of anti-TNF biologics, we attempted to analyse the mRNA expression profiles in peripheral blood samples from RA patients taken at multiple time points during treatment with a chimeric anti-TNF-α monoclonal antibody, infliximab. In this report, we show that several sets of genes were closely correlated with inflammatory response markers, like CRP, while other gene groups with unique kinetics were also identified. Interestingly, the kinetics of the expression patterns of several genes was clearly discordant between responders and non-responders to infliximab. This information may help to personalize therapeutic strategies using anti-TNF biologics.

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Materials and methods

Patients

To fulfil the ACR criteria for RA [21, 22], patients had to be at least 18 yrs of age, have an ACR functional class of I–III and should have been receiving MTX ≥6 mg/week for a minimum of 3 months and a stable dose for at least 6 weeks at the time of study enrolment. Patients meeting these criteria were recruited at the Division of Rheumatology/Clinical Immunology, Saitama Medical Center, Saitama Medical University, Japan. All patients must have had inadequate control of RA symptoms, as defined by a combination of ≥6 swollen joints, ≥6 painful joints and an ESR ≥28 mm/h or a CRP level of 20 mg/l, while receiving a stable MTX dose that at its maximum did not exceed the 2.5 × upper normal limit of a liver enzyme test. The exclusion criteria for infliximab treatment were based on the Japanese guidelines for the use of infliximab [23]. In addition to these guidelines, RA patients with other collagen-vascular disease complications were excluded from the study, with the exception of patients with secondary SS. The diagnosis of SS was made according to the American–European criteria [24]. Informed consent was obtained from all patients, in accordance with the Helsinki protocol.

Study procedures and evaluations

Infliximab (Remicade; Tanabe Seiyaku, Osaka, Japan) was infused at a dose of 3 mg/kg at 0, 2, 6 and every other 8 weeks thereafter in combination with the ongoing administration of MTX at a dose >6 mg/week. The doses of MTX, other DMARDs, NSAIDs and steroids were fixed throughout the entire study period. IA steroid injections were not permitted. Blood samples were collected immediately before the first intravenous injection of infliximab and 2, 14 and 22 weeks after the first injection to measure the serum protein and autoantibody levels and for mRNA profiling.

The patients were examined at every hospital visit they made to receive an infusion. The ACR core set of variables, including the number of swollen joints, the number of painful joints, physician's global assessment on a visual analogue scale (VAS) of 0–100 mm, patient's global assessment of disease activity on a scale of VAS (0–100 mm), duration of morning stiffness (minutes) and pain on a scale of VAS (0–100 mm), was evaluated. Disease activity was assessed using the ACR criteria for 20% improvement (ACR20), the ACR50, the ACR70 and the disease activity score of 28 joints (DAS28) [25].

The serum CRP level was measured using an LPIA CRP kit (Mitsubishi Chemical Iatron, Tokyo, Japan), the MMP-3 level was measured using an MMP-3 ELISA kit (Daiichi Chemicals, Tokyo, Japan) and the RF level was measured using an N-Latex-RF kit (Dade Behring, Tokyo, Japan). ANA was measured using indirect immunofluorescence on Hep-2 (MBL, Nagoya, Japan) and was defined as positive if the observed titre was ×80 or greater than ×80. Anti-SS-A(Ro), anti-SS-B(La) and anti-U1RNP were measured using an ELISA (MBL) and were defined as positive if the observed units were greater than the cut-off units. Human anti-chimeric antibody (HACA; anti-infliximab antibody) was measured using an ELISA (Immunodiagnostik, Bensheim, Germany) and was determined as positive according to the manufacturer's instructions. Briefly, the diluted serum samples (×200) were added onto infliximab-coated microtitre plates and incubated overnight at 4°C After extensive washing with buffer, infliximab conjugated with horseradish peroxidase was added and the samples were incubated, followed by extensive washing. Finally, the substrates were added to the plates and the optical density at 450–620 nm was measured.

Hand and foot X-rays were obtained at baseline, and two expert readers scored the images according to the previously reported method of vdH-Sharp [26].

The design of this study was approved by the ethical committees of Saitama Medical University (No. 173), and all the patients provided their written informed consent at the time of enrolment in the study.

Preparation of RNA from blood

Samples (2.5 ml × 2) of whole blood were drawn into PAXgene RNA tubes (Qiagen, Hilden, Germany), and the total RNA was extracted and purified according to the tube manufacturer's instructions. The quantity of RNA obtained from the extraction step was assessed using a NanoDrop ND-1000 instrument (Nano Technologies, Wilmington, DE, USA). The quality of the extracted RNA was determined using a Bioanalyser 2100 (Agilent Technologies, Palo Alto, CA, USA); the ribosomal RNA 28S/18S ratio was verified to be >1.3 in all the experiments.

Preparation of the cDNA microarray

We designed and prepared a low-density cDNA microarray for mRNA expression profiling in whole blood. Genes for this microarray were selected from the public database of SAGE (serial analysis of gene expression) results (http://133.11.248.12/; homepage of the Department of Molecular Prevent Medicine, School of Medicine, The University of Tokyo) for activated blood cells, such as T cells, dendritic cells, monocytes and macrophages [27–29]. We also incorporated findings from a high-density oligo-chip assay (U-95 GeneChip; Affymetrix, Santa Clara, CA, USA) that were obtained using peripheral blood mononuclear cells (PBMCs) isolated before and after infliximab treatment. A total of 747 genes were spotted onto SuperAmine (Telechem International, Sunnyvale, CA, USA) in quadruplicate along with positive and negative control genes, as described previously [29]. For most of the genes, each cDNA was designed to be ~500–600 bp and to be within ~1 kb from the 3′-poly A tail. All cDNAs for the microarray probe were cloned into the pGEM vector (Promega, Madison, WI, USA). All clones for the capture probe were sequenced and validated by comparison with the GenBank sequence.

To confirm the sensitivity and reproducibility of this customized DNA microarray, we used PBMCs stimulated *in vitro* with lipopolysaccharide (LPS) at a concentration of 5 μg/ml and monitored the mRNA expression for up to 16 h using this microarray. At the same time, an ELISA was employed to measure the TNF-α produced in the culture supernatant. A significant increase in TNF-α in the LPS-stimulated culture supernatants was confirmed, indicating that this system worked well (data not shown). While we did not detect any significant change in TNF-α mRNA before stimulation, the amount of transcript sharply increased with LPS stimulation, as detected using the microarray system (a 25-fold increase, compared with an unstimulated control, at 2 h), followed by a gradual decrease to the basal level. Following LPS stimulation, other transcripts, such as plasminogen activator inhibitor-II (PAI-II) and IFN-induced cellular resistance mediator protein A (MxA), showed a biphasic response, increasing initially and then returning to a lower level. Other transcripts, such as orsomucoid-1 and IL-10, showed a continuous increase for at least 16 h. Similar results were obtained in five repeated experiments, confirming the sensitivity and reproducibility of this system.

Reference RNA

Reference RNA was established from a mixture of whole blood (drawn into PAXgene tubes) RNA samples from healthy volunteers. The extracted total RNA, which was certified to be of sufficient quality using the Agilent RNA chip, was amplified using the MessageAmp aRNA kit (Ambion, Austin, TX, USA) to generate amplified RNA (aRNA). An external non-human artificial RNA (a *Caenorhabditis elegans* Y49G5B fragment) was spiked into the reference aRNA to distinguish it from the sample aRNA.

Preparation of sample RNA, labelling, hybridization and scanning

Total RNA (1 μg) from the patients was transcribed and amplified into aRNA using the MessageAmp aRNA kit (Ambion, Austin, Texas), according to the manufacturer's instructions. Next, an external control RNA mixture [lambda DNA (LD), a baculovirus glycoprotein gene (GP) and a *Renilla* luciferase gene (RL); 9 μg each] were added to both the sample and the reference aRNA. The sample and reference aRNAs were then labelled with Cy5-dUTP and Cy3-dUTP (PerkinElmer, Boston, MA, USA), respectively, using a SuperScript II kit (Invitrogen, Carlsbad, CA, USA) and random hexamers (TaKaRa, Kyoto, Japan). Competitive hybridization of Cy3-labelled reference and Cy5-labelled sample cDNA on the microarray was performed using a chamber system (Agilent Technologies, Palo Alto, CA, USA), according to the method described by Khodursky *et al.* [30]. The slides were scanned five times with five different power ranges using a ScanArray 5000 (PerkinElmer, Boston, MA, USA). For further statistical analysis, the data were converted from TIFF image data to signals using ImaGene software (BioDiscovery, El Segundo, CA, USA). The data files for the five scans were merged to establish a single representative data set for each gene (patent pending, PCT/JP03/06677). The Cy5 (patient sample)/Cy3 (reference sample) ratio for each mRNA signal was calculated after global Lowess normalization [31].

Real-time PCR

The primer and probe sets for IFN-inducible double-stranded RNA-activated protein kinase (PKR) (forward, CCTGTCCT CTGGTTCTTTTG; reverse, TGTCAGGAAGGTCAAATCTG; probe, CTACGTGTGAGTCCCAAAGCAAC) and IFN-inducible transmembrane 9–27 (forward, CCGTGCCCGACCATGT; reverse, CCCAGACAGCACCAGTTCAA; probe, TGGTCCC TGTTCAACACCCTCT) were prepared according to TaqMan Gene Expression Assays (a pre-formulated assay; Applied BioSystems, Foster City, California, USA). The probes were fluorescently labelled with 5-carboxyfluorescein (FAM; reporter) and tetramethyl-rhodamine (TAMRA; quencher) dye systems (Applied BioSystems, Foster, CA, USA). To determine the relative amount of RNA, standard curves were generated for each primer-probe set using the same reference aRNA as the microarray. TaqMan fluorescence-based quantitative real-time PCR was performed in 384-well plates on an ABI Prism7900HT sequence detection system, according to the manufacturer's protocol. According to the manufacturer's instructions, reverse transcription and amplification were accomplished in a single step using the TaqMan EZ RT-PCR core reagent (Applied BioSystems), 10 ng of patient aRNA, 100 nM of each primer and 200 nM of TaqMan probe labelled at the 5′-end with FAM and at the 3′-end with TAMRA. The reaction was performed using the following sequence: 2 min at 50°C, 30 min at 60°C, 5 min at 95°C and 50 cycles of 20 s at 95°C followed by 1 min at 62°C.

Statistics

Statistical analyses were performed using JMP software version 6.0 (SAS Institute, Tokyo, Japan), unless otherwise specified. The Wilcoxon/Kruskal–Wallis test was used for non-parametric comparisons between subgroups. Chronological changes in the same items, such as microarray data (Cy5/Cy3 ratio), the serum CRP level and the DAS28 score in responders and non-responders, were initially analysed using an equal variance *f*-test. If the *P*-value from the *f*-test was <0.05, the Welch *t*-test was used for comparisons. Differences were considered significant when the *P*-value was <0.05.

Results

Patient characteristics and changes in clinical parameters

The baseline characteristics and the response to infliximab at 22 weeks in the 18 RA patients enrolled in this study are shown in Table 1. Four of the 18 patients had early stages of RA, with durations of <2 yrs. No significant differences in the clinical parameters were observed between the responders and the non-responders, although the CRP and MMP-3 levels were higher and the RF titres and vdH-Sharp scores were lower in the responders than in the non-responders in this patient population. During the infliximab treatment, significant changes in the populations of various blood cell types, including white blood cells, neutrophils, eosinophils, basophils, monocytes and lymphocytes, were not observed (data not shown). To assess the clinical response to infliximab treatment, we calculated the ACR response rate and the DAS28 based on the clinical data obtained at each visit. The 18 RA patients were then categorized as responders ($n=8$) or non-responders ($n=10$) based on their ACR50 response criteria at week 22, as shown in Table 1. The ACR50 response rate to infliximab was 8/18 (44.4%), which was higher than that observed in a Japanese clinical trial in a 3 mg/kg infliximab group at 14 weeks (30.2%) [32]. The reductions in the serum CRP levels and the DAS28 scores after the first infusion of infliximab were similar between the responders and the non-responders. However, these values increased with subsequent infusions in non-responders, while they remained at low levels in responders (Fig. 1). HACA directed against infliximab was detected in 3 of the 10 non-responders but was not detected in the 8 responders. HACA positivity in patients with ACR0, ACR20, ACR50 and ACR70 was 14.3, 30.0, 0 and 0%, respectively.

cDNA microarray analysis of peripheral blood cells in RA patients treated with infliximab

We first selected genes that were differentially expressed by >1.5-fold between the patient and the reference control samples for at least one sampling point (see Supplementary Fig. 1, available as supplementary data at *Rheumatology* Online). Next, we searched for genes with statistically significant differences in expression between responders and non-responders to infliximab.

Approximately 15% of the total genes were found to have a >1.5-fold change, compared with the reference value, at one or more time points during 22 weeks of infliximab therapy. These genes included those for ribosomal proteins, proteins related to degradation or apoptosis (e.g. caspase and proteasome components) and proteins related to metabolism (e.g. folate receptor). As shown in Table 2, 18 genes that were differentially expressed between responders and non-responders during the monitoring period were successfully identified. Interestingly, the top ten genes in Table 2 were IFN-related. Prior to treatment, IFN-related genes were up-regulated in >50% of the RA patients; regulation was normalized in some of the patients, as shown in Fig. 2A. When the patients were classified as responders or non-responders, distinct patterns were observed at 14 weeks and similar patterns, but consisting of three clusters, at 22 weeks, but this was not obvious at 2 weeks. It is interesting to note that one of the two responders in the non-responder cluster at 14 weeks (marked as violet) was moved to the responder cluster at 22 weeks, but another responder (marked as pink) remained at the non-responder cluster at 22 weeks. Also, one non-responder in the responder cluster at 14 weeks (marked as yellow) remained in the responder cluster at 22 weeks. The responders were characterized by continued suppression to the normal level, while expression in the non-responders returned to the baseline level (Table 2). The expressions of IFN-related genes, including IFN-inducible gene family (transmembrane 2), IFN-induced

TABLE 1. Characteristics of enrolled patients at baseline

No.	Sex	Age	Duration (months)[a]	DAS28	CRP (mg/dl)	MMP-3 (ng/ml)	RF (IU/ml)	ANA(x)	anti-SSA (IU/ml)	anti-SSB (IU/ml)	anti-RNP (IU/ml)	Total Sharp score	MTX (mg/week)	Previous DMARDs[b]	PSL (mg/day)	Response at 22 w	ACR response at 22 w	DAS28 at 22 w	CRP at 22 w (mg/dl)	MMP-3 at 22 w (ng/ml)	HACA at 22 w
1	F	61	83	6.91	5.8	578	55	320	0.4	5.8	4.1	31	8	1	12.5	R	ACR70	1.74	0.1	129	−
2	F	63	75	7.69	8.2	286	523	80	0	1.9	1.4	100	10	1	10	NR	ACR0	5.61	7.4	433	−
3	F	67	311	6.52	2.7	93	220	80	0.1	1.7	1.6	189	8	2	10	NR	ACR0	4.82	0.7	144	−
4	F	51	132	5.88	2	77	41	80	2.7	19.3	7.4	41	8	1	4	R	ACR50	3.1	1	164	−
5	M	34	4	6.02	4.1	140	555	160	76.9	5.3	3.1	36	8	1	8	NR	ACR20	3.69	2.3	271	+
6	F	51	60	6.93	7.8	346	12	160	76.9	5.3	3.1	36	13	2	0	NR	ACR0	5.85	8.2	638	+
7	F	58	30	6.72	2.4	156	530	80	0.4	5.9	2.6	75	8	2	9	NR	ACR20	4.11	1.7	305	−
8	F	52	132	6.36	4.7	209	0	160	0.4	4.1	3.9	26	10	0	10	R	ACR70	2.41	0.1	94	−
9	F	58	178	4.88	2.1	520	702	160	25.1	2.5	1.2	345	6	5	7	NR	ACR20	3.89	1.2	498	+
10	F	66	44	7.74	4	367	784	640	109.3	4.4	5.6	51	6	4	0	NR	ACR0	7.35	4.3	1030	+
11	F	60	122	4.55	2.8	135	19	2560	1.9	6.1	9.6	104	8	3	3	R	ACR70	1.88	0.2	40	−
12	F	52	5	5.09	2.5	185	22	80	0.3	2.5	2.4	31	10	1	5	R	ACR50	2.50	0.1	99	−
13	F	45	73	6.65	1.8	153	80	80	0.3	3.2	3.5	115	8	3	7.5	NR	ACR0	4.62	0.2	200	−
14	F	60	243	6.47	2.7	115	108	80	1.3	3.6	3	290	6	3	5	NR	ACR0	4.72	2.2	314	−
15	M	42	70	4.04	0.6	422	62	(-)	0.3	6.4	2.3	137	8	4	7.5	NR	ACR0	4.73	2.2	355	−
16	M	62	17	6.82	7.6	514	340	80	0.3	6.1	2.4	132	8	2	6	R	ACR50	4.47	2.6	420	−
17	F	52	255	5.72	3.3	191	845	160	0.1	5	3.9	188	8	2	0	R	ACR50	3.46	0.4	76	−
18	F	53	13	6.51	13.6	1460	0	320	10	2	4	88	8	1	8	R	ACR70	3.44	0.4	330	−
Total		Mean	54.8	102.6	6.2	4.4	330.4	272.1				111.9	8.3	2.1	6.3			4.02	2.0	307.8	16.7%
		s.d.	8.6	91.5	1.0	3.2	323.4	301.2				91.3	1.7	1.3	3.7			1.44	2.4	243.1	
Responder		Mean	55.4	94.9	6.0	5.3	418.6	165.3				80.1	8.5	1.4	6.1			2.88	0.6	168.9	0%
		s.d.	4.7	84.6	0.8	3.9	457.7	296.8				58.9	0.9	0.9	4.0			0.92	0.9	134.4	
Non-responder		Mean	54.4	108.8	6.4	3.6	259.8	357.6				137.4	8.1	2.7	6.4			4.94	3.0	418.8	30%
		s.d.	11.0	100.8	1.2	2.5	148.7	290.9				106.9	2.1	1.3	3.7			1.09	2.7	258.5	

[a]Period from diagnosis to the start of infliximab treatment; [b]Number of previous DMARDs used before infliximab infusion. RF, MMP-3 and HACA were measured as described in the Materials and methods section. R, Responder; NR, Non-Responder.

cellular resistance mediator protein B (MxB), IFN-α-inducible peptide (6–16) gene, PKR and IFN-inducible transmembrane protein 9–27, showed similar patterns (Table 2 and Fig. 2B), suggesting that these genes may be regulated by a common key molecule. Indeed, some of these genes, including PKR, 2′,5′-AS (OAS1), ISG-56 and IFI-6-16, have an IFN-stimulated response element (ISRE) in their promoter regions [33, 34].

To confirm the data obtained using the customized cDNA microarray, we also analysed mRNA expression using real-time PCR. Figure 3 shows the results of real-time PCR data for IFN-inducible transmembrane protein 9–27 in responders and non-responders. The microarray data and the real-time PCR data in Fig. 3 are comparable, confirming the results. Expression profiling showed that the marked up-regulation of genes related to inflammation was suppressed to the reference level after treatment with infliximab. As shown in the left half of the Fig. 2B, a significant decrease in the up-regulated genes at baseline was obtained at 2 weeks after infliximab treatment in most of the patients, regardless of whether they were responders (closed circles) or non-responders (open circles). The decrease in the up-regulated genes in the responders was maintained throughout the treatment period after 2 weeks. Interestingly, the up-regulation of IFN-related genes re-appeared in parallel with the flare in DAS28 and serum CRP levels after 2 weeks in the non-responders (Fig. 1). Changes in the DAS28 level were strongly correlated with the expression of IFN-related genes, as shown in the right half of Fig. 2B.

In addition to the IFN-related genes, several genes, such as the AP-1-associated adaptor complex subunit (γ-adaptin) and some chemokines, showed unique kinetics patterns. As shown in Fig. 4D, the gene expression of the AP-1-associated adaptor complex subunit (γ-adaptin) was increasingly up-regulated during the late phase of infliximab treatment in responders, in contrast to the pattern observed in the IFN-related genes. Interestingly, significant differences in the expression of CX3CR1, IL2RB and chemokine ligand 4 genes (Fig. 4A–C) as well as TNF-related genes (data not shown) were observed between responders and non-responders at baseline.

Discussion

In this report, we utilized a customized microarray to monitor the kinetics of mRNA expression profiles in peripheral blood samples from RA patients during treatment with infliximab, a chimeric monoclonal antibody against TNF-α. Although synovial biopsy samples reflect the consequences of inflammation at a specific joint and the therapeutic response to anti-TNF biologics more directly [19, 35], obtaining such samples, particularly from multiple sites and at multiple time points, is difficult. Thus, we focused on the use of peripheral blood samples.

Variability in microarray data arises from many sources, including sampling, RNA amplification and labelling, and the hybridization conditions. In particular, reliable procedures for blood sampling and the separation of PBMCs are indispensable for minimizing *ex vivo* changes in mRNA expression [36, 37]. Since the PAXgene RNA tube blood-drawing system can reduce or eliminate problems arising during handling or storage [38–40], we used this system and obtained an excellent quality and quantity of mRNA in whole blood cells from RA patients. Our initial experiments using LPS-activated PBMCs indicated that our customized microarray detected an increase in the expression of TNF-α mRNA prior to that of IFN-related genes, including MxA and 2′,5′-AS (OAS1). Since the corresponding protein levels measured by ELISA also changed in a fashion similar to the changes in mRNA expression detected using the customized microarray, this array seems to be useful for detecting biologically meaningful changes in mRNA levels.

Open-labelled studies tend to exhibit a bias in the interpretations of responses towards a favourable outcome, particularly

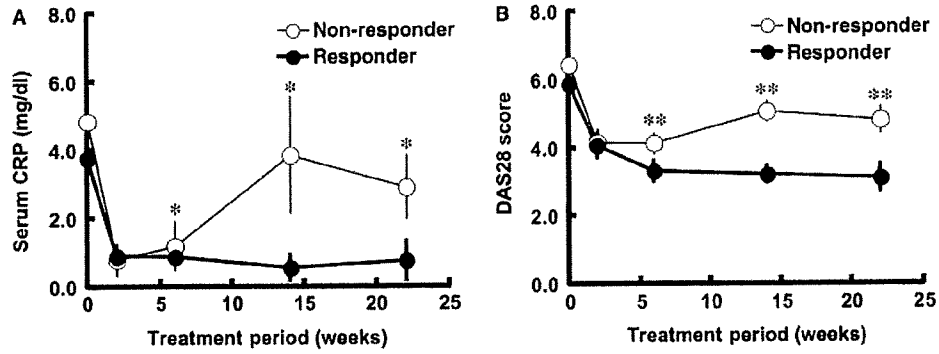

FIG. 1. Time course of CRP and DAS28 in RA patients during infliximab treatment. Serum CRP level (**A**) and the DAS28 score (**B**) in the responder ($n=8$) and non-responder groups ($n=10$). $^{*}P<0.05$ and $^{**}P<0.01$ between responders (filled circle) and non-responders (open circle).

TABLE 2. Genes differentially expressed between the responders and non-responders

Gene name	GenBank Accession No.	NM_ID	Before treatment		2 weeks		14 weeks		22 weeks	
			Responder	Non-responder	Responder	Non-responder	Responder	Non-responder	Responder	Non-responder
MxA (IFN-induced cellular resistance mediator protein) (MX1)	M30817	NM_002462	2.16±0.36	1.76±0.25	1.54±0.21	1.23±0.19	3.11±0.90	1.27±0.16	2.99±0.62	1.15±0.16
2,5-AS-1 40/46 kDa (OAS1)	D00068	NM_002534	1.95±0.38	1.18±0.17	1.39±0.32	0.91±0..10	2.12±0.52	0.90±0.07*	2.17±0.53	0.90±0.11
2,5-AS-2 71 kDa (OAS2)	M87434	NM_016817	1.72±0.33	1.26±0.14	1.24±0.20	1.10±0.09	2.34±0.49	1.29±0.14	2.44±0.56	1.24±0.17
RIG-G, IFN-induced protein with tetratricopeptide repeats 3 (IFIT3)	U52513	NM_001549	2.14±0.43	1.63±0.27	1.42±0.27	1.39±0.28	2.20±0.52	1.04±0.10	1.86±0.33	0.99±0.12
ISG-56K(IFN-inducible 56 kDa protein) (IFIT1)	X03557	NM_001548	2.28±0.58	1.68±0.35	1.43±0.34	1.42±0.30	1.84±0.38	0.85±0.09*	1.92±0.39	1.03±0.21
MxB (IFN-induced cellular × M resistance mediator protein) (MX2)	M30818	NM_002463	1.74±0.19	1.61±0.18	1.21±0.10	1.14±0.13	1.66±0.23	1.05±0.11*	1.65±0.23	0.91±0.09
IFN-inducible dsRNA-activated protein kinase (p68kinase) (PRKR)	M35663	NM_002759	2.33±0.22	1.96±0.10	1.89±0.25	1.66±0.18	2.35±0.31	1.46±0.05*	2.72±0.32	1.53±0.08
IFI-6-16, IFN-α inducible (G1P3)	BC011601	NM_002038	1.78±0.30	1.51±0.26	1.21±0.197	1.26±0.18	2.79±0.77	1.37±0.11	2.67±0.49	1.52±0.21
IFN-inducible transmembrane protein 9–27 (IFITM1)	J04164	NM_003641	1.62±0.16	1.44±0.31	0.98±0.10	1.00±.12	2.07±0.35	1.16±*0.12	1.75±0.18	1.13±0.10
Inducible gene family (transmembrane protein 2 (1-8D) (IFITM2)	X57351	NM_006435	1.98±0.33	1.87±0.41	1.15±0.14	1.15±0.16	1.82±0.26	1.09±0.12*	1.52±0.19	1.06±0.11
HLA-class II, DQ α 1 (HLA-DQA1)	M34996	NM_002122	1.10±0.25	0.53±0.10	1.33±0.32	0.63±0.15*	1.07±0.26	0.66±0.15	0.91±0.13	0.68±0.17
TRIP14 (thyroid receptor interactor), exon 3 (OASL)	L40387	NM_003733	1.43±0.20	1.10±0.08	1.10±0.10	0.98±0.08	1.83±0.27	1.17±0.07*	1.51±0.19	1.13±0.08
Rearranged immunoglobulin mRNA for μ-heavy chain enhancer and constant region (IGHM)	X58529		0.99±0.13	0.71±0.16	1.40±0.25	0.88±0.21**	0.87±0.18	0.68±0.18	0.73±0.12	0.66±0.18
Tis11d (ZFP36L2)	U07802	NM_006887	0.49±0.07	0.49±0.06	0.54±0.09	0.59±0.05	0.36±0.05	0.54±0.08	0.36±0.05	0.57±0.05
Signal recognition particle subunit 9 kDa protein (SRP9), binding Alu sequence	U20998	NM_003133	0.51±0.07	0.55±0.10	0.64±0.09	0.71±0.06	0.32±0.05	0.43±0.07	0.36±0.05	0.56±0.07
IL-2 receptor β-chain (p70–75) (IL2RB)	M26062	NM_000878	0.66±0.04	0.97±0.09	0.78±0.05	0.95±0.09	0.67±0.04	1.03±0.14	0.70±0.07	0.89±0.06
CX3C chemokine receptor-1 (CX3CR1)	BC028078	NM_001337	0.73±0.07	0.97±0.08	0.66±0.04	0.83±0.09	0.58±0.03	0.89±0.12	0.66±0.06	0.77±0.08
AP-1 clathrin adaptor complex, sigma 1B subunit (AP1S2)	AB015320	NM_003916	1.04±0.08	0.98±0.06	1.02±0.07	1.22±0.07**	1.30±0.12	1.65±0.12*	1.32±0.11	1.75±0.14

The values represent the mean Cy5/Cy3 ratios±S.E. from 8 to 10 patients. $^{*}P<0.05$ and $^{**}P<0.01$ between the ACR0–20 and the ACR50–70 groups at the same time point.

when the responders are judged according to ACR20 criteria. Instead, we used the ACR50 response to measure the response rate in this study because this measure is thought to better reflect the patients' levels of satisfaction [41, 42]. Indeed, when we categorized the responders according to the ACR20 criteria, we did not observe a clear difference in the CRP level or the DAS28 score between the responders and non-responders. In turn, when we used ACR50 as the definition of response, significant differences in the CRP level and the DAS28 score were observed between the two groups. Thus, the patients were grouped as responders or non-responders based on their ACR50 response, similar to the protocol previously used to group therapeutic

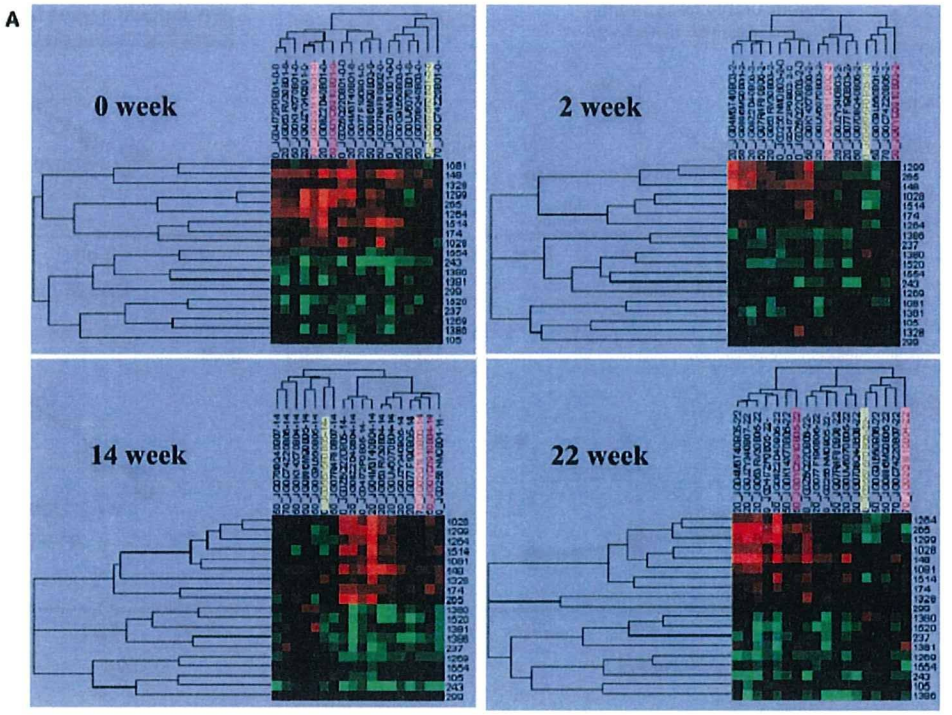

FIG. 2. (A) Cluster analysis of gene expression profile at 0, 2, 14 and 22 weeks after infliximab treatment. Responders are shown in green and non-responders are shown in red in the tree-cluster. The gene set used here was as follows (JGS No., symbol, GenBank ID): 105: CDC42 (NM_001791), 148: PKR (NM_002759), 174: ISGF3G (NM_006084), 237: IL2RB (NM_000878), 243: STMN1 (NM_005563), 265: IFIT1 (NM_001548), 299: CD68 (NM_001251), 1028: IFITM1 (NM_003641), 1081: BST2 (NM_004335), 1264: MxB (NM_002463), 1269: EIF3S10 (NM_003750), 1299: IFIT3 (NM_001549), 1328: SELP (NM_003005), 1380: GZMA (NM_006144), 1381: GZMB (NM_004131), 1386: CX3CR1 (NM_001337), 1514: IFITM2 (NM_006435), 1520: CCL4 (NM_002984), 1554: PTMA (NM_002823). $^{*}P<0.05$ and $^{**}P<0.01$ between responders (filled circle) and non-responders (open circle).

responses to MYX and etanercept [11]. Recently, accumulating evidence has demonstrated that the combination of anti-TNF biologics and MTX protects against joint destruction in all RA patients, irrespective of their clinical response, for up to 2 yrs of observation [43–45]; this finding raises the question as to whether a non-responder phenotype really exists and whether this phenotype is stable, particularly in terms of the response to joint damage. In these studies, the mean change in the joint score of the RA patients treated with anti-TNF plus MTX was almost zero. However, several groups of patients were examined, including those with the progression of radiographic joint damage, those with no significant change and those with an improvement. In this regard, it may be interesting to apply this microarray analysis to the prediction of patients with variable radiographic changes.

We observed a clear, statistically significant difference in the kinetics of IFN-related genes during infliximab treatment between the responders and the non-responders. One may wonder whether this difference is a consequence of the successful sustained inhibition of TNF-α production in responders, but not in non-responders. The sustained inhibition of TNF-α production depends, in part, on the trough level of the serum infliximab concentration, which has been reported to be correlated with the efficacy of infliximab [46–48]. The serum trough level of infliximab can be determined by several factors, such as the dose of infliximab, immunoglobulin clearance and HACA [49] in individual patients. Although the necessary trough level of infliximab should be determined in the future, HACA was positive at 22 weeks for 3 out of 10 non-responders, while it was not detected in 8 responders, suggesting that HACA and the subsequent promotion of infliximab clearance may lead to the inefficient inhibition of TNF-α production. Nevertheless, it is important to note that TNF-α itself does not differ significantly between responders and non-responders to infliximab at 22 weeks, indicating that other mechanisms may also be involved in this process.

Interestingly, the IFN signature was originally reported in the expression profiles of PBMCs from patients with SLE, but not from patients with RA [50, 51]. In this study, the IFN signature was also observed in a subset of RA patients, and TNF-α blockade caused an immediate and significant reduction in IFN-related gene expression, although the level of the increase in IFN-related genes in RA patients was 2–3 times that in healthy individuals, while the increase in patients with SLE was much higher. Recently, TNF-α has been shown to regulate type 1 IFN. Juvenile idiopathic arthritis patients treated with anti-TNF exhibited the IFN signature, which is responsible for the seroconversion to anti-dsDNA antibody, and lupus-like clinical manifestations developed in RA patients receiving anti-TNF therapy [52]. While these studies were performed in a limited number of juvenile patients, other reports have identified patients with the IFN signature in a subset of SLE patients with renal disease and anti-RNA-binding proteins like anti-RNP/Sm and anti-Ro/La, but not with anti-dsDNA antibodies. Furthermore, a significant association between high IFN-α scores and the absolute counts of lymphocytes in PBMC samples was observed, partly because of lymphocytopenia [53]. In this regard, SS complications may confer an IFN signature as a result of lymphocytopenia and anti-Ro/La positivity. Among our 18 RA patients, 4 patients had been diagnosed as having secondary SS. However, the IFN signature was not limited to the RA patients with secondary SS, ruling out this possibility. An elevated level of serum IFN-α may contribute to the IFN signature seen in patients with SLE [54]. Finally, it has been reported that the serum IFN-α level is elevated in patients with RA [55] and that IFN-α treatment for hepatitis C complications in RA patients induced or aggravated RA [56]. Again, it should be noted that quantitative RT-PCR showed no significant

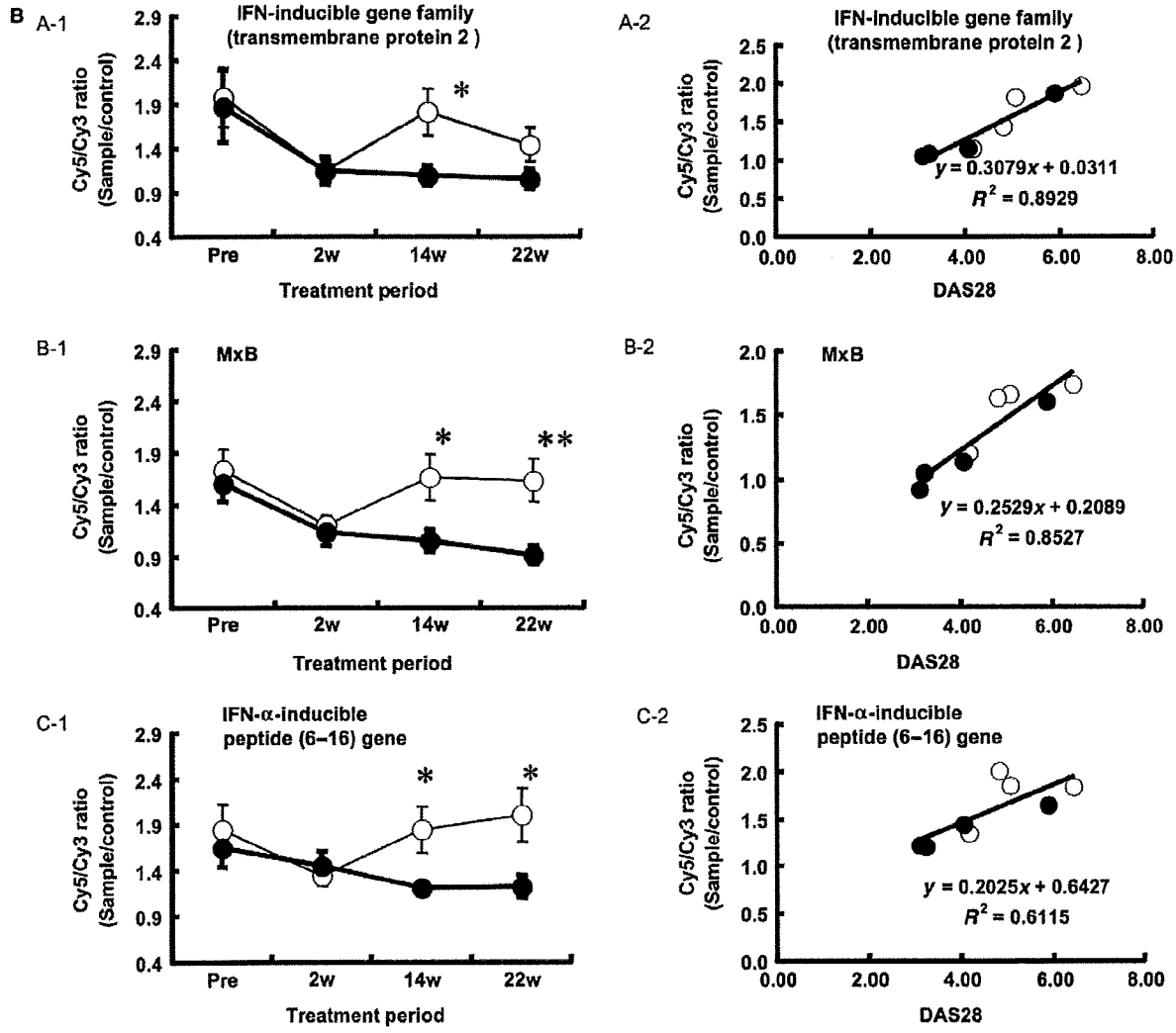

FIG. 2. (Continued). (B) Changes in the expression of IFN-related genes in RA patients during infliximab treatment as determined by microarray analysis and correlation between the expression and DAS28 score. In the left panel, the typical time courses for the expression of INF-related genes are shown, including IFN-inducible gene family (transmembrane protein 2), MxB and INF-α-inducible peptide (6–16) gene in responders (filled circle) ($n=8$) and non-responders (open circle) ($n=10$). The data represent the mean values ± S.E. In the right panel, the correlations between the level of individual gene expressions and the DAS28 score are shown. The *Y*-axis represents the microarray fold-change, and the *X*-axis represents the DAS score. $^{*}P<0.05$ and $^{**}P<0.01$ between responders (filled circle) and non-responders (open circle).

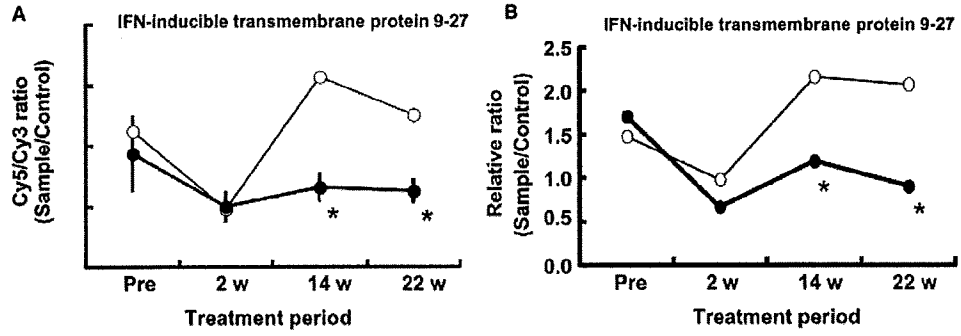

FIG. 3. Changes in the expression of IFN-inducible transmembrane protein 9–27 mRNA in RA patients during infliximab treatment, as determined by microarray and semi-quantitative real-time PCR. The expression of IFN-inducible transmembrane protein 9–27 (IFITM1) mRNA was determined by microarray (**A**) and by real-time PCR (**B**). The results are depicted by the symbols for responders (filled circle) and non-responders (open circle). $^{*}P<0.05$ between responders (filled circle) and non-responders (open circle).

increases in IFN-γ or IFN-α/β genes in peripheral blood samples, as demonstrated in this study. The mechanism responsible for the sustained inhibition of the IFN signature in responders and its reappearance in non-responders remains to be clarified, and such knowledge will likely identify new therapeutic targets for RA.

The AP-1-associated adaptor complex subunit is a member of the vesicle budding protein family and plays a role in protein

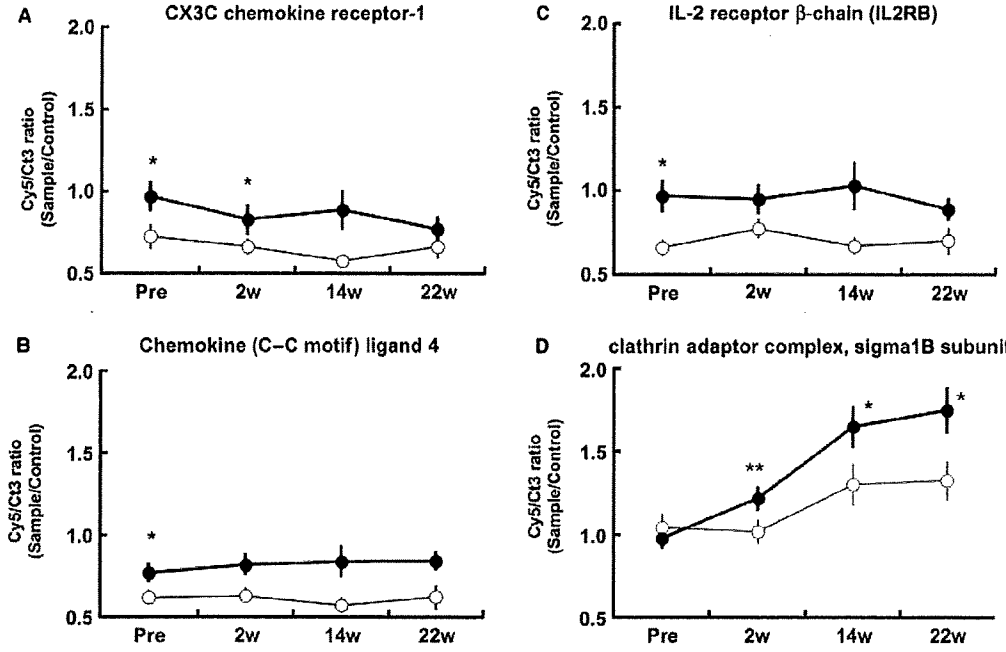

FIG. 4. Changes in the expression of cytokines, chemokines, and AP-1 mRNA in RA patients during infliximab treatment, as determined by microarray. X-axis shows treatment period. The expressions of CX3C chemokine receptor-1 (**A**), chemokine (C–C motif) ligand 4 (**B**), IL-2 receptor β-chain (**C**) and AP-1 clathrin adaptor complex, sigma 1B subunit (**D**) mRNA were determined by microarray in responders (filled circle) and non-responders (open circle). The data are represented as the mean ± S.E. $^{*}P < 0.05$ and $^{**}P < 0.01$ between responders (filled circle) and non-responders (open circle).

transport between membrane compartments in receptor-mediated endocytosis [57]. This gene was up-regulated in the responder groups during the later phase of treatment. We suspect that TNF receptor recycling is activated in patients during the later stages of infliximab treatment, but the biological significance of this process is currently unclear. The expression of CD28 (data not shown) and ribosomal proteins also exhibited the same pattern as the AP-1-associated adaptor complex subunit.

We found that HLA-DQA1 was up-regulated at baseline, and its expression did not change significantly during the course of treatment. Although SNP analyses indicated a correlation between responsiveness to infliximab and the expression of HLA-B-associated transcript 2 and some other antigens [12], recent studies have shown that the shared epitope is correlated with disease severity, but not with the response to anti-TNF biologics [13]. On the contrary, the expression levels of HLA-DRB1 alleles and some other loci have been reported to be associated with the response to etanercept [11], supporting our results.

A recent microarray analysis of mononuclear cells separated from the peripheral blood of RA patients identified 20 genes that were significantly correlated with a favourable response to infliximab [20]. Interestingly, most of the genes listed were not related to the pathophysiological condition and were not TNF-α targets, but two genes, PTPN12 and MUSTN1, are regulated by the TNF-α/nuclear factor-κB pathway [20]. The 20 genes that were listed are not the same as those identified in this report, with the exception of HLA class II. This discrepancy may be due to differences in the samples (mononuclear cells *vs* whole blood cells), array systems, statistical analysis methods, patient ethnic and treatment backgrounds, criteria for responders and so on. Consistent with their results, we did not observe a clear change in TNF-related gene expression in peripheral blood cells after infliximab treatment. Our microarray may not have been sufficiently sensitive, although an *in vitro* experiment demonstrated a definite ability of the system to detect changes in TNF-α expression. Since the decay of the TNF-α gene transcript was rapid and occurred after <12 h in the *in vitro* experiment, the sampling timing may be critical for detecting significant changes *in vivo*.

In conclusion, we showed that a customized microarray could be used to monitor mRNA expression in peripheral blood cells from RA patients treated with an anti-TNF biologic, infliximab. Our results support the strategy of using a downsized, customized microarray and whole blood cell samples to identify potential responders to TNF inhibitors and to identify new molecular targets by analysing the expression profiles of non-responders.

Rheumatology key messages

- Using a customized microarray, we demonstrated unique genes in responders and non-responders to infliximab in RA.
- It is useful to predict responders and identify new targets by analysing gene profiles in non-responders.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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Special Report

EXPERT REVIEWS

Current Japanese regulatory situations of pharmacogenomics in drug administration

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Pharmacogenomics (PGx) has the potential impact to improve drug-development efficiencies and proper usages of drugs in clinical practice. However, in order to translate PGx into practical applications, multidisciplinary challenges, such as cost and time in development, processes of genomic biomarker qualification, PGx test availabilities and reimbursements, and education on PGx, still remain in clinical, pharmaceutical and regulatory settings. Japanese regulatory bodies for drug approval (i.e., Ministry of Health, Labour and Welfare and Pharmaceutical and Medical Devices Agency) have been taking proactive actions, both internally and internationally, toward translating PGx from bench to bedside. In this article, we summarize the current situations and projects in regulatory implementations of PGx in drug administrations in Japan, including activities to promote PGx-based drug/device developments and therapies. Moreover, we also discuss the future tasks for utilization of PGx in drug evaluations and clinical practices.

KEYWORDS: drug development • drug evaluation • genomic biomarker • Japan • Ministry of Health Labour and Welfare • Pharmaceutical and Medical Devices Agency • pharmacogenomics

The inter-individual variability in drug responses, such as bioavailabilities, efficacies and adverse effects of drugs, is a major issue to determine an optimal dose in drug developments and clinical practices [1–3]. Mainly, there are two potential factors, extrinsic ethnic factors and intrinsic ethnic factors, influencing drug responses [101]. Examples of former factors include the social and cultural aspects, such as medical practice, diet, use of tobacco, use of alcohol, exposure to pollution and socio–economic status, and latter factors include genetic polymorphism, age, gender, height, weight, lean body mass, body composition and organ dysfunction. Among these factors, inherited factors are one of the most important potential factors that affect the drug responses. Especially, genetic variations in genes relating to processes of drug actions, such as drug metabolizing enzymes, drug transporters, drug receptors and other pharmacological targets, are of even greater importance since accumulated knowledge of human genomic variation is being used for drug evaluations [4]. For example, it is reported that the genetic polymorphisms in the genes encoding β-adrenergic receptor and cytochrome P450 (CYP)2C9 are potential factors to explain the ethnic variability in drug response [5].

Pharmacogenomics (PGx) is defined as the study of variations of DNA and RNA characteristics related to drug responses by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guidance: Topic E15 [102]. PGx has the potential to improve drug-development efficiencies and proper usages of drugs in clinical practices. In other words, promises of PGx lie in the possibility to reduce an attrition rate of drug development and to optimize a dose setting of a drug for individual patients [6–8]. Especially, maximizing effectiveness and minimizing the risks of drugs by introducing PGx into drug development will enable drug development to be more successful, since regulatory decisions of drug approvals have been made based on their benefit:risk ratio.

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In this article, we summarize the current situations and projects in regulatory implementations for PGx for drug evaluations in Japan, including activities to promote PGx-based drug/device developments and therapies. In addition, we also discuss future tasks for the utilization of PGx in drug evaluations and clinical practices.

Current activities to promote drug/device developments using PGx in Japan

Ministry of Health, Labour & Welfare activities

The Ministry of Health, Labour and Welfare (MHLW) has released notifications to promote drug/device developments using PGx. As shown in TABLE 1, the MHLW has taken proactive actions aiming to translate PGx from bench to bedside.

In June 2001, two notifications entitled *Guidance on Clinical Pharmacokinetics Studies of Pharmaceuticals* and *Guidance on Methods of Drug Interaction Studies* were published [9,10]. These two notifications show the basic concept related to a clinical pharmacokinetics study and drug-interaction study for new drug application. The former guidelines note that if a genetic polymorphism is likely to affect individual differences in pharmacokinetics, a sponsor is recommended to select subjects with or without specific genetic factors, based on objective criteria, such as genotyping tests [9]. In the 'Questions and Answers' section, it mentions that ethical issues should be taken into consideration to perform genetic examinations, and that genetic polymorphisms can be identified either by genotyping or phenotyping. However, in the case of genotyping, the genotype that clearly relates to metabolic activity should be used. It is also mentioned that, if a probable percentage of target genetic polymorphisms is very low in Japanese populations, data from foreign populations are expected to provide valuable information. The latter guidelines describe that, in case a polymorphically expressed enzyme significantly affects metabolism of the investigational drug, a sponsor is recommended to study drug interactions, considering phenotypes and/or genotypes of individual subject [10]. Thus, based on these notifications, the MHLW has encouraged sponsors to conduct studies using PGx, which can assess potential impacts of genetic polymorphisms of drug metabolizing enzymes on pharmacokinetics of individual subjects in clinical trials.

In March 2005, *Submission of Information to Regulatory Authorities for Preparation of Guidance on the Use of Pharmacogenomics in Clinical Studies* was published [11]. This notification encouraged sponsors to voluntarily submit a list of information to the MHLW on planned, ongoing, and past PGx clinical trials. The purpose of this notification is to collect all available information regarding PGx clinical trials to correctly understand situations of PGx activities in drug development.

Table 1. Notifications and reports related to pharmacogenomics issued by Ministry of Health, Labour and Welfare.

	Issued date	Notifier
Notification		
Guidance on Clinical Pharmacokinetics Studies of Pharmaceuticals	June 2001	PFSB/ELD
Guidance on Methods of Drug Interaction Studies	June 2001	PFSB/ELD
Submission of Information to Regulatory Authorities for Preparation of Guidance on the Use of Pharmacogenomics in Clinical Studies	March 2005	PFSB/ELD
Request to cooperate in research regarding severe cutaneous adverse reactions	June 2006	PFSB/SD
Terminology in Pharmacogenomics (ICH-E15 guideline)	January 2008	PFSB/ELD and PFSB/SD
Points to Consider for Evaluating Genotyping Platforms Based on DNA Chips	April 2008	PFSB/ELD
Reports		
Pharmaceuticals and Medical Devices Safety Information No. 219 – Future Prospect of Pharmacogenomics (1)	November 2005	PFSB/SD
Current Situations and Future Tasks for Utilization of Pharmacogenomics in Drug Evaluation	March 2007	The Japanese Society of Clinical Pharmacology and Therapeutics*
Pharmaceuticals and Medical Devices Safety Information No. 235 – Future Prospect of Pharmacogenomics (2)	April 2007	PFSB/SD

*The Japanese Society of Clinical Pharmacology and Therapeutics published the report by request from Ministry of Health, Labour and Welfare.
MHLW: Ministry of Health, Labour and Welfare; PFSB/ELD: Pharmaceutical and Food Safety Bureau/Evaluation and Licensing Division; PFSB/SD: Pharmaceutical and Food Safety Bureau/Safety Division.

Submitted lists include following information related to the trials, a purpose of the study, a phase of the study, the name of country where the study is performed, a target gene, a target disease, races of these individuals, subject numbers, methods for genetic tests and sample storage processes. In total, the information of 179 clinical trials from 22 industries were submitted based on the notification. Through this process, accumulated knowledge and experiences are intended to be used for taking appropriate regulatory measures, such as the establishment of guidelines.

In March 2007, the Japanese Society of Clinical Pharmacology and Therapeutics published the report entitled *Current Situations and Future Tasks for Utilization of PGx in Drug Evaluation* by the request of the MHLW [12]. The report summarized the current knowledge and usefulness of PGx and identified future tasks that should be taken into consideration in regulatory sciences to promote PGx utilization in drug development and clinical practices in Japan. In order to further introduce PGx in drug development in Japan, five issues were identified:

- Establishment of general guidelines regarding PGx applications in clinical trials
- Clarification of PGx data handling in a common technical document (CTD) for a new drug application
- Considering potential strategies for promoting drug/device codevelopments for utilizing PGx
- Establishment of general guidelines regarding genomic biomarkers in drug development
- Establishment of general guidelines regarding clinical trial designs using PGx

A guideline entitled *Points to Consider for Evaluating Genetic Tests Based on DNA Chips* was also released in April 2008 [13]. The purpose for the preparation of this guideline was to facilitate considerations by industries and accelerate regulatory reviews regarding issues in *in vitro* diagnostics (IVD) devices. It summarizes important points to obtain reliable data genotyped by DNA chips and its dedicated devices, including software. This guideline is expected to will mutually promote the development of genetic tests and PGx-based drug developments in Japan.

Regulatory approaches relating to PGx are expanding globally and many regulatory authorities have published guidelines or concept papers [9,10,13–15,103,104]. In this new field, international regulatory collaborations are important to avoid unnecessary works for regulatory measures, such as establishment of guidelines related to PGx. ICH will be an appropriate forum for the purpose of harmonizing regulatory approaches in PGx, since more than 50 regulatory guidelines of drugs in the quality, safety, efficacy and multidisciplinary fields have already been harmonized among the regions. The MHLW is currently working on harmonizing regulatory approaches relating to PGx in the ICH, in addition to the domestic activities described previously. In November 2007, the ICH established the E15 guideline entitled *Definitions for Genomic Biomarkers, Pharmacogenomics, Pharmacogenetics, Genomic Data and Sample Coding Categories* [102]. This is the first guideline for the ICH to adopt in this emerging science area and it will be a basis for future ICH guidelines relating to PGx. In January 2008, the ICH-E15 guideline was officially notified and implemented in Japan [15]. The guideline will help to make common understanding of PGx issues, not only in ICH regions, but also in non-ICH regions, by avoiding either conflicting use of terms in regulatory documentation and guidelines, or inconsistent interpretation among regions and any bodies relating to PGx, such as regulatory authorities, sponsor companies and ethical committees.

Pharmaceutical & Medical Devices Agency activities

The Pharmaceutical and Medical Devices Agency (PMDA) is an independent regulatory agency from the MHLW and is responsible for scientific reviews for the approval of drugs and medical devices and for safety monitoring after approval.

In September 2005, the Pharmacogenomics Discussion Group (PDG) was established in the PMDA to manage PGx issues from regulatory stand points with cooperation of the MHLW. Missions of the PDG are described as follows: share PGx data, information and knowledge, discuss regulatory issues relating to PGx, keep consistency of PMDA decisions among offices/reviewers and promote appropriate drug development using PGx. The PDG currently consists of 21 members from various offices in PMDA, such as the Office of New Drugs, Office of Biologics, Office of Medical Devices, Office of Review Administration, Office of Safety and Office of Compliance and Standards. The PDG has not only had internal meetings on a regular basis, but also more than 15 informal meetings with pharmaceutical companies and academia over the last 2.5 years to understand updated scientific knowledge relating to PGx and to identify practical issues for PGx utilization in drug/device developments in Japan. Major topics in the informal PDG meetings were drug/device development strategies using PGx and interpretation of PGx data, including genomic biomarkers. For example, issues regarding processes of sample storages in PGx studies, including sample coding and sample withdrawal, codevelopment strategies of devices with drugs and interpretations of preliminary PGx data, were discussed. Increased interactions between pharmaceutical companies/academia and the PMDA at the informal PDG meetings will help and promote PGx applications in drug development and clinical practices. Recently, the PDG joined the US FDA and European Medicines Agency (EMEA) joint Voluntary Genomic Data Submission briefing meeting as an observer [105]. Strengthening collaborations with other international regulatory agencies, such as the FDA and EMEA are important to promote appropriate PGx use in drug development and clinical practices and harmonization of regulatory approaches in PGx.

Current PGx implementations for drugs approved in Japan

Availabilities of PGx information in package inserts on approved drugs in Japan

Package inserts are the most fundamental tools to provide information on approved drugs to healthcare professionals and promote the proper use of drugs. Package inserts of drugs approved in Japan are posted on the information page on the PMDA website [106]. We searched this publicly available PMDA website to investigate how much information on genomic biomarkers is included in package inserts of new drugs that were reviewed by the Drug Committees of the Pharmaceutical Affairs and Food Sanitation Council of the MHLW and were approved from fiscal year 2002 to fiscal year 2006 in Japan. The criteria for a selection were to include at least one of 12 items (i.e., single nucleotide polymorphism, genome, genomics, metabolic pathway, genotype, polymorphism, poor metabolizer, extensive metabolizer, metabolizer, pharmaco-, genetic and variation). Numbers of accumulated package inserts including PGx information have been increasing year by year during the period (Figure 1). Approximately 16% of the package inserts (32 out of 199 package inserts) included the information related to PGx in the 2006 fiscal year.

The PGx information in the package inserts was classified into four groups according to the type of genomic biomarker as follows; metabolizing enzyme, virus and bacterium, pharmacological target and others (Table 2). Within the 32 package inserts including PGx information, the most common type was 'virus and bacterium', which consists of 17 products, such as hepatitis B virus (HBV), hepatitis C virus (HCV) and HIV. For example, efficacy results based on genotypes of HCV in clinical trials were included in the package inserts of ribavirin and pegylated (peg)-IFN-α_{2a}. Similarly, the resistance of drugs to certain HIV genotypes was described in the package inserts for anti-HIV drugs, such as emtricitabine and lamivudine–abacavir sulfate. The second most popular type after 'virus and bacterium' was 'metabolizing enzyme' such as CYP. For example, information on the differences of pharmacokinetic parameters, such as AUC, C_{max} and elimination half-life ($t_{1/2}$) between extensive metabolizers and poor metabolizers were described in the package inserts of letrozole for *CYP2A6*, tolterodine tartrate for *CYP2D6* and proton-pump inhibitors, such as omeprazole, sodium rabeprazole and lansoprazole for *CYP2C19*. 'Pharmacological target' information was the third most popular group, but was limited in the field of anticancer drugs, such as imatinib mesylate, for patients with KIT (CD117)-positive metastatic malignant gastrointestinal stromal tumors and tamibarotene for patients with *PML-RAR-α* gene positive acute promyelocytic leukemia. A recent study showed similar tendency to our finding (i.e., the genes coding for drug metabolizing enzymes and viral genes was the major PGx information included in the package inserts of drugs approved in the USA) [16].

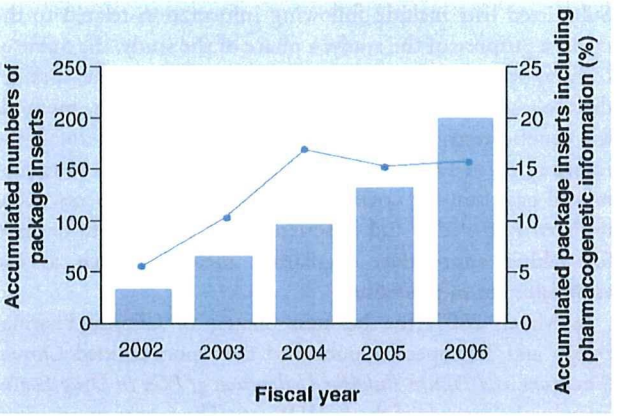

Figure 1. Trends in information relating to pharmacogenomics in package inserts of drugs approved in Japan. Bars represent the accumulated numbers of package inserts of new drugs that were reviewed by the Drug Committees of the Pharmaceutical Affairs and Food Sanitation Council of the Ministry of Health, Labour and Welfare and were approved in Japan from fiscal year 2002 to fiscal year 2006. Generic and over-the-counter drugs are not included. Circles and line are the accumulated percentages of package inserts of the drugs including information related to pharmacogenomics.

According to the degree of requirement for testing of the genomic biomarker, PGx information was classified into three types: 'test required', 'test recommended' and 'information only' (Table 2). Each type of information relating to the requirement of testing was calculated as one case, thus, 50 cases of information were identified in the 32 package inserts because two or three cases were included in a package insert. Types of genomic biomarkers and relationships with PGx test requirement status are shown in Figure 2. PGx-related information of metabolizing enzyme only showed 'information only', which provides the allele frequency of *CYP* mutant gene (i.e., *CYP2A6*, *CYP2C9*, *CYP2C19* and *CYP2D6*) and the relationship between genotypes and pharmacokinetics parameters. Within the PGx-related information of 'virus and bacterium', 9% of the information was 'test required', asking genotyping of the virus to be performed before starting drug administrations in order to achieve a desired antiretroviral therapeutic effect. For example, one of the indications of peg-IFN-α_{2a} was limited to patients infected with HCV genotype I and/or II. The percentages of 'test recommended' and 'information only' regarding 'virus and bacterium' were 39 and 52%, respectively. Within the PGx-related information of 'pharmacological targets', 60% of the cases were categorized as 'test required', asking for diagnostic genetic tests to confirm the existence of genomic biomarker before starting administrations of anticancer drugs (i.e., imatinib mesylate, arsenic trioxide and tamibarotene). Although relationships between clinical outcome and PGx-based prescribing information in package inserts of drugs have not been studied extensively , the PGx-based information relating to 'test required' and 'test recommended' in package inserts can be contributed for promoting to conduct proactive PGx studies.

Table 2. Drugs including PGx information in package inserts in Japan.

Genomic biomarker	Drug name (nonproprietary name)	Requirement status of testing for the genomic biomarker
Metabolizing enzyme		
CYP2A6	Letrozole	Information only
CYP2C9	Candesartan Cilexetil	Information only
	Celecoxib	Information only
CYP2C19	Omeprazole–clarithromycin–amoxicillin	Information only
	Clarithromycin	Information only*
	Sodium rabeprazole‡	Information only
	Voriconazole	Information only
	Lansoprazole	Information only
CYP2D6	Gefitinib	Information only
	Tolterodine tartrate	Information only
Virus/bacterium		
Cytomegalovirus	Valganciclovir hydrochloride	Information only
Enterococcus faecium *Enterococcus faecalis* *Staphylococcus aureus* *Staphylococcus epidermidis*	Linezolid	Information only
Hepatitis B virus	Adefovir dipivixil	Recommended
	Lamivudine	Recommended
	Entecavir hydrate	Recommended
Hepatitis C virus	Peg-IFN-α_{2a}‡	Required
	IFN-α_{2b}	Recommended
	Peg-IFN-α_{2b}	Recommended
	IFN-β	Recommended
	Ribavirin	Required
HIV	Atazanavir sulfate	Recommended
	Tenofovir disoproxil fumarate	Information only
	Fosamprenavir calcium hydrate	Recommended
	Lamivudine–abacavir sulfate	Recommended
	Emtricitabine	Recommended
	Emtricitabine-tenofovir disoproxil fumarate	Recommended
Pharmacological target		
KIT expression, Philadelphia chromosome presence	Imatinib mesylate	Required
PML/RAR-α fusion gene presence	Arsenic trioxide	Required
	Tamibarotene	Required
Others		
Protein C deficiency	Human-activated protein C§	Required

*Information on coadministration with clarithromycin and sodium labeprazole.
‡Counted twice because approved indications, dosage and administration were partially changed during the period.
§Indications of human-activated protein C are deep venous thrombosis, acute pulmonary thromboembolism and fulminant purpura due to congenital protein C deficiency. Protein C deficiency is classified into 'others' because this genomic biomarker is directly concerned with the hereditary disorder.

Moreover, as use of PGx test and qualification of genomic biomarkers are actively studied in drug development, the availability of PGx-based prescribing information in package inserts could be expanded in the near future.

Current situations of PGx tests covered by National Health Insurance

Reimbursement of the medical fee is one of the important factors to translate validated genomic biomarker from the research environment to clinical use [17–19].

Fortunately, Japan has the National Health Insurance (NHI) system, which applies to all Japanese citizens. In this system, parts of medical costs, including medication and the use of medical devices, are reimbursed. As described in the previous section, some package inserts of approved drugs include information on PGx tests as a requirement or a recommendation before starting drug administrations, specifically in the field of antivirus and anticancer drugs. In these fields, several PGx tests are already available that can be covered by the NHI. Major PGx tests related to leukemia, malignant tumor and infectious disease, which are covered by the NHI over the past 5 years in Japan, are summarized in Table 3. [20].

For example, PGx testd for EGF receptor (*EGFR*) gene mutation in lung cancer patients was covered by the NHI in June 2007. It has been reported that efficacy of gefitinib for the treatment of non-small-cell lung cancer depends on genetic variants of *EGFR*, and *EFGR* mutation rate is higher in Japanese than that in Caucasian individuals [21,22]. Consequently, many commercial clinical laboratories are currently available in Japan for providing the service of analysis of *EGFR* gene mutations, such as exon 19 deletions (e.g., *E746-A750* deletion), *T790M* substitution in exon 20, and *L858R* substitution in exon 21. In the near future, as implementation of PGx test for *EGFR* gene mutation widely spreads in clinical practices, data of *EGFR* gene mutation as a genomic biomarker relating to drug responses will be further accumulated.

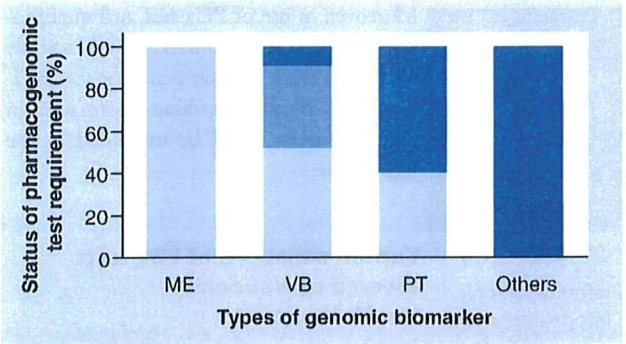

Figure 2. Relationships between types of genomic biomarker and status of pharmacogenomics (PGx) test requirements in the package inserts of drugs approved in Japan. Percentages of PGx test-requirement status are shown in each type of genomic biomarker included in the package inserts of new drugs that were reviewed by the Drug Committees of the Pharmaceutical Affairs and Food Sanitation Council of the Ministry of Health, Labour and Welfare, and were approved in Japan from fiscal year 2002 to fiscal year 2006. Dark, mid and light blue bars are 'test requirement', 'test recommended ' and 'information only', respectively. Total numbers of information cases relating to PGx tests in ME, VB, PT and others are 11, 33, five and one, respectively.
ME: Metabolizing enzyme; PT: Pharmacological target; VB: Virus and bacterium.

Furthermore, to allow drug selections and reselections of antiretroviral drugs in order to avoid drug inefficacy and drug resistance, a genotyping test for HIV drug resistances was covered by the NHI in April 2006. It is reported that drug resistance is closely related to virological failure and poorer prognosis in drug-experienced patients [23]. The prevalence of drug resistant HIV-1 strains is also related to global availability of antiretroviral drugs [23,24]. Therefore, a PGx test would be of growing importance in maintaining optimal efficacy of the anti-HIV drugs for the long-term periods.

As described previously, the several NHI-covered PGx tests are widely available in the fields of cancer therapy and antiretroviral therapy in Japan.

MHLW activities to identify potential factors associated with drug-related severe adverse reactions

The notification entitled *Request to Cooperate in Research Regarding Severe Cutaneous Adverse Reactions* was issued by the MHLW in June 2006 (Table 1) [14]. This notification encourages pharmaceutical companies to cooperate to the exploratory research of genomic biomarkers in order to predict drug-related severe cutaneous adverse reactions, such as Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), in the National Institute of Health Science (NIHS). In case a serious cutaneous adverse event, such as SJS and TEN, occurs and is recognized, the pharmaceutical companies will voluntary inform the event to the NIHS based on the notification and, additionally, report adverse drug reactions to the PMDA based on the Pharmaceutical Affairs Law where necessary (Figure 3). The incidence frequencies of SJS and TEN are extremely low (approximately one or two patients per 1 million people per year) [25]. The pathological mechanisms underlying the onset of SJS and TEN are not fully

Table 3. Major reimbursable PGx tests in Japan.

Genomic biomarker	Requirements for reimbursement	Date to be covered by NHI
PGx tests related to leukemia and malignant tumor		
Her2/neu (erbB2)	FISH assay for selection of HER2 overexpressing breast cancer patients appropriate for HER2 monoclonal antibody therapy	April 2003
Major bcr-abl mRNA	TMA assay to quantify major bcr-abl mRNA in hematocyte	November 2004
EGFR mutations	Test to detect *EGFR* mutations in patient with lung cancer	June 2007
KIT mutations	Test to detect *KIT* mutations in patient with gastrointestinal stromal tumor	June 2007
Wilms tumor-1 mRNA	Real time RT-PCR assay to quantify wilms tumor-1 mRNA for diagnostic aid and follow-up in patients with acute myelocytic leukemia	November 2007
PGx tests related to infectious disease		
Mutations in HBV precore, mutations in HBV core promoter	PCR assay to detect mutations in HBV precore and core promoter in plasma	July 2003
Mutations in HIV	Test to detect mutations in HIV for anti-HIV drugs selection and reselection	April 2006

EGFR: Epidermal growth factor receptor; FISH: Fluorescence *in situ* hybridization; HBV: Hepatitis B virus; HER2: Human epidermal growth factor receptor 2; NHI: National Health Insurance; PGx: Pharmacogenomics; RT-PCR: Reverse transcription polymerase chain reaction; TMA: Transcription-mediated amplification.

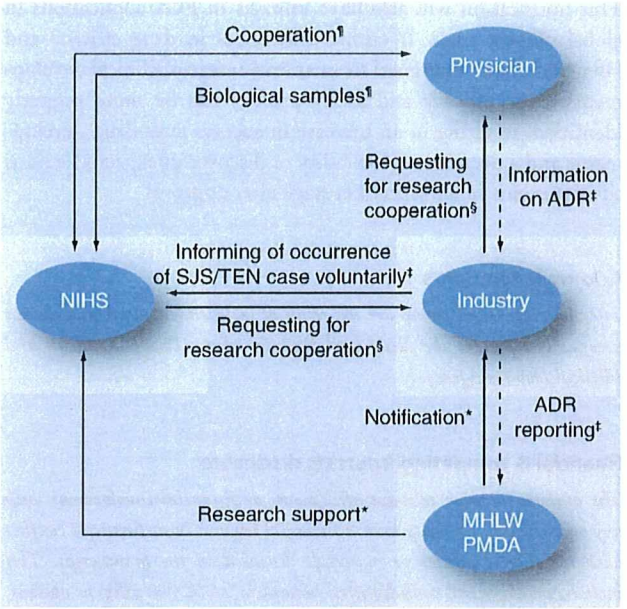

Figure 3. Framework of research regarding severe cutaneous adverse reactions in NIHS. Dotted arrows represent the flow of information on report of ADR to the PMDA based on the Pharmaceutical Affairs Law. Solid arrows represents the scheme related to the research regarding severe cutaneous adverse reaction, including SJS and TEN, in the NIHS.
*Request to cooperate in research regarding severe cutaneous adverse reactions: Safety Division, Pharmaceutical and Food Safety Bureau, MHLW, Notification No. 0615001 [14].
‡In case a serious cutaneous adverse event, such as SJS and TEN, occurs and is recognized, the industry will voluntarily inform the NIHS based on the notification, and additionally report on ADR to PMDA based on the Pharmaceutical Affairs Law where necessary.
§Then, to the physicians who diagnosed the case, the NIHS requests for cooperation to the research via industries.
¶The NIHS will conduct genetic analysis only after the physician agrees to the request.
ADR: Adverse drug reaction; MHLW: Ministry of Health, Labour and Welfare; NIHS: National Institute of Health Sciences; PMDA: Pharmaceuticals and Medical Devices Agency; SJS: Stevens–Johnson syndrome; TEN: Toxic epidermal necrolysis.

established, although the involvement of immune mechanisms has been suggested. A recent study suggested the strong genetic association between HLA and drug-induced SJS/TEN [26,27]. For example, 100% association of carbamazepine-induced SJS and TEN with *HLA-B*1502* has been reported in 44 Han Chinese patients [28]. Meanwhile, such association is not obtained in patients of European ancestry [29]. It is suggested that although the HLA region may contain important genes for SJS and TEN, the *HLA-B*1502* allele may not be a universal marker for this adverse reaction. Roles of *HLA-B*1502* for predicting SJS/TEN risks in Japanese populations are not currently clear, but allele frequency of *HLA-B*1502* was 0.1% in unrelated healthy Japanese individuals [30], which is lower than that in Han Chinese populations (10–15%) [107]. In drug development, it is difficult to study about an extremely rare adverse event, such as a drug-induced SJS or TEN. Therefore, a cooperate research to explore genomic biomarkers related to severe adverse drug reactions will be useful to accumulate basic information in order to establish proper actions based on PGx in Japan.

Pharmaceuticals & Medical Devices Safety Information

With the objective of providing the latest information and safety topics for the safer use of drugs and medical devices to healthcare professionals, the MHLW has compiled commentaries and notices when major revisions based on important case reports on severe adverse reactions were made in package inserts. A digested form, *Pharmaceuticals and Medical Devices Safety Information*, was also published bimonthly from June 1973 and then monthly from June 2001 [108]. As of February 2008, 244 digests have been published and disseminated to healthcare professionals in Japan. In two digests published in November 2005 (no. 219) and April 2007 (no. 235), the MHLW has featured on the future prospects of PGx (Table 1). The digests summarized the domestic and international situations of updated practical usage of PGx. In the no. 219 digest, it was described that the MHLW encourages industries to develop IVD devices for genotyping *UGT1A1* mutant genes, which have been reported to have a potential role in irinotecan-induced neutropenia [31]. In irinotecan therapy, some alleles of *UGT1A1*, such as *UGT1A1*6* and **28*, have been suggested to have clinical impacts in Japanese populations [32–34]. It is expected that PGx-based dose adjustments of irinotecan in clinical practices will be popularized when the IVD device for the genotyping of these *UGT1A1* mutant alleles is approved in Japan.

Although PGx information for intended dose adjustments based on genetic profiles is currently limited in package inserts in Japan, the more information that is included, the more IVD devices for validated genomic biomarkers are approved.

To promote the appropriate use of PGx in clinical practices, the PGx information in package inserts should be properly interpreted and understood. However, in Western countries and Japan, the lack of knowledge regarding PGx among healthcare professionals has been reported [35–37]. Therefore, adequate education and supply of proper information to people who prescribe, dispense and use drug products are indispensable. From this viewpoint, PGx information in the digest will contribute greatly to establish the basis of PGx-based medicine in clinical practices in Japan.

Expert commentary

Looking at current PGx implementation for approved drugs, utilization of PGx has been expanding recently in Japan. To achieve the goal of PGx utilizations, strengthening cooperation/collaboration among regulatory agencies and other stakeholders, such as industries, academia and healthcare professionals, and further international harmonization of PGx regulatory approaches, are critical and essential.

Five-year view

As described previously, some genomic biomarkers that are useful in a certain population may not be useful in other populations (e.g., the association between carbamazepine-induced SJS/TEN and *HLA-B*1502*). Therefore, in order to apply PGx to drug development and usage of drugs in clinical settings appropriately, it should be taken into consideration that allele frequencies of the genomic biomarkers may be different among populations. During the drug development period especially, it will be very important to examine from an early stage whether ethnic differences in a genomic biomarker have any impact on drug efficacy and safety.

In the future, drug developments will be more globalized and PGx in drug developments will be more practically applied and implemented. In such situations, PGx data obtained in global clinical trials including various populations who may have various ethnicities are very important in order to properly evaluate PGx utilities in drug administration. To promote simultaneous global drug developments including Japan, the MHLW published the final notification of points to consider document entitled *Basic Principles on Global Clinical Trials* last September [38]. This notification will also have impacts in PGx applications in global clinical trials. If ethnic differences in drug efficacy and safety have been examined from an early stage of clinical developments, drug efficacy and safety profiles can be more properly identified, resulting in an increase in success rates drug developments and a decrease of possibility to discover unexpected serious adverse events in a postmarket stage after approval.

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Key issues

- Over the past 5 years (from fiscal year 2002 to fiscal year 2006), of the approved drugs in Japan, approximately 16% of the package inserts (32 out of 199 package inserts) included the information related to pharmacogenomics (PGx).
- Several PGx tests for genomic biomarkers (e.g., *EGFR* mutation, *KIT* mutation and mutations in HIV) are covered by National Health Insurance, and widely available in the field of cancer therapy and antiretroviral therapy in Japan.
- PGx implementations, in terms of information in package inserts and reimbursable PGx tests, could rapidly expand in Japan.
- The Pharmacogenomics Discussion Group of the Pharmaceuticals and Medical Devices Agency has played an important role in promoting PGx utilization in Japan, as well as international harmonization of PGx regulatory approaches.
- The Ministry of Health, Labour and Welfare has taken action to promote PGx utilization in Japan, such as publishing guidelines, disseminating PGx information and supporting PGx researches.
- Establishing more guidelines relating to PGx based on the report from the Japanese Society of Clinical Pharmacology and Therapeutics will be necessary in order to further introduce PGx in drug developments in Japan.
- To achieve the goal of PGx utilizations, strengthening cooperation/collaboration among regulatory agencies and other stakeholders, such as industries, academia and healthcare professionals, and international harmonization of PGx regulatory approaches, are critical and essential.
- PGx applications in global drug development will also be important to properly evaluate PGx utilities in drug administration and can contribute to increased efficiency of drug development.

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- •• of considerable interest

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国際共同治験の基本的考え方について***

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1. 医薬品開発の現状

1.1 臨床開発の失敗

世界の医薬品開発の中心はアメリカです．したがって，アメリカでは多くの治験が行われていますが，様々な理由で失敗するものも多くあります[1,2]．どの領域でも程度の差こそあれ，臨床試験の少なくとも1割は失敗しているとの分析もありますが，実際にはもっと失敗している可能性があります．

臨床開発が失敗している原因についても，様々な理由がありますが，時間の経過に伴って失敗の原因が変わってきています．例えば，新手法であるマイクロドージングの説明をする際に良く用いられる表現として，以前はPKの問題によって開発を失敗していたので，この部分をきちんと評価するためにマイクロドージングを用いると，早い段階でPKの特性の優れたものを選べるため，PKの問題による失敗は減らせるといった例があります．

マイクロドージングに限った話ではありませんが，科学の進歩によって吸収性が良くないものや生体内の安定性が悪いものは早いうちに予測できるようになり，そういった化合物は早いうちに排除できます．例えば，1991年と2000年で比べますと，PKなどの問題で失敗する例はかなり減少しました[2]．

一方で，有効性について差が出ず，開発を中止してしまうものや，あるいは後で毒性の問題が現れて失敗してしまうもの，毒性といっても恐らく臨床的な副作用が背景にあると思われるもので，それをきっかけに様々な動物実験を行ってみると思わぬ毒性があることが判明してしまうものや，あるいは臨床試験と並行して追加で行っている長期の動物実験で予測できない毒性が現れることもあります．

こうした様々な原因によって開発効率が落ちています．実際に臨床開発に入ったものが徐々に脱落し，申請してから承認される間に更に脱落します．化合物を探索する段階から考えますと，5000分の1，1万分の1の割合でしか残らないともいわれており，開発効率の悪いことが非常に問題となっています．

ただし，化合物を選ぶ部分に関しては，ベーシックサイエンスの進歩に期待するものですので，我々が現在関心を持っているのは，臨床開発での脱落を何とか減らすことで，開発を進める間に，日本が乗り遅れないようにどうやって開発の中に入っていくか，場合によっては日本がリードすることもあっても良いと考えています．

1.2 国内企業の医薬品開発の動向

国内企業の医薬品開発の動向を見た場合，最近10年間では海外先行で開発するものが着実に増え，相対的に国内先行の割合が減少しています[3]．

これはある意味，日本の製薬企業が海外にも拠点を持ち，海外での開発展開を開発のオプションの中にきちんと入れるようになり，真の意味でグローバル化が日本の製薬企業においても進んできたことを表しているといえます．

しかし一方で，日本国内の臨床開発環境が劣悪であることが明らかとなるにしたがって，条件の整った海外での開発を先行させるようになっているとの指摘も確かにあります．この問題は日本での臨床開発の今後の行方を予測する上で非常に深刻な，懸念

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