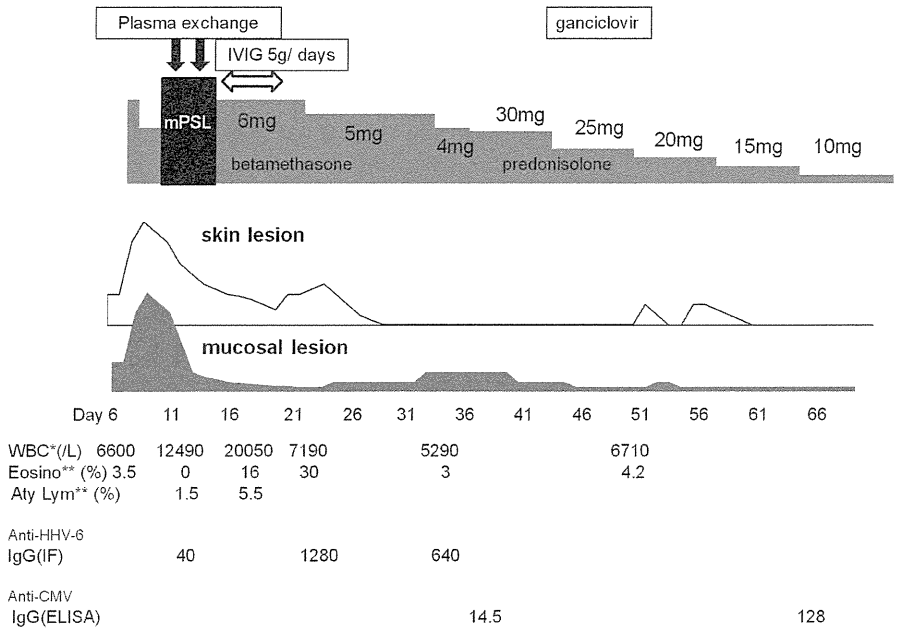

Fig. 2. Clinical course. Lesions on the patient's skin and oral mucosa recurred several times, with high fever, increase in atypical lymphocytes, eosinophilia, and human herpesvirus (HHV)-6 and cytomegalovirus (CMV) reactivation. *WBC: white blood cell count; **Eosino: eosinophils; ***Aty Lym: atypical lymphocytes.

B*58:01 positivity. It was concluded that the type of ADR was TEN with HHV-6 reactivation. Symptoms consistent with TEN were as follows: (*i*) severe mucosal lesions, (*ii*) widespread skin detachment, and (*iii*) histopathological findings of epidermal necrosis and subepidermal blisters. Some symptoms consistent with DRESS were also observed as follows: (*i*) high fever, (*ii*) acute skin rash, (*iii*) peripheral blood abnormalities, such as leukocytosis, eosinophilia, and atypical lymphocytosis. In addition, reactivation of HHV-6 and CMV as well as several recurrences of skin rash were consistent with DIHS. Although serious internal organ involvement was not observed, pulmonary oedema and a mild increase in serum creatinine were observed.

HHV reactivation is rarely observed in patients with SJS/TEN. Only a few cases have been reported as SJS/TEN associated with HHV-6 reactivation (8, 9), and TEN with HHV-7 reactivation (10). These patients did not show frequent recurrence and haematological abnormalities as observed in our patient. Since our patient showed clinical features of TEN accompanied with some symptoms of DRESS with HHV-6 and CMV reactivation, we conclude that viral reactivation is involved in the clinical course. Clinicians should consider sequential testing for HHV-6 with prolonged SJS/TEN, especially when induced by drugs known to be causative of DIHS, including allopurinol and anti-epileptics.

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INVITED ARTICLE

Pharmacogenetics of cutaneous adverse drug reactions

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ABSTRACT

Drug-induced hypersensitivity reactions are of major medical concern because they are associated with high morbidity and high mortality. In addition, individual patients' reactions are impossible to predict in each patient. In the field of severe cutaneous adverse drug reactions (cutaneous ADR) such as Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug-induced hypersensitivity syndrome (DHIS) or drug rash with eosinophilia and systemic symptoms (DRESS), major advances have recently been gained through studies of an association between HLA alleles and drug hypersensitivity induced by specific drugs. The results of these pharmacogenomic studies allow prediction of the risk of adverse reactions in patients treated with certain drugs, including carbamazepine and other aromatic antiepileptic drugs, allopurinol and abacavir. However, different ethnic populations show variations in the genetic associations. A strong association between carbamazepine-induced SJS/TEN and HLA-B*1502 has been found in Southeast Asian patients but not in Caucasian and Japanese patients. Moderate associations between aromatic amine anticonvulsants and other HLA alleles have been proposed in Japanese patients. In contrast, HLA-B*5801 was found to be associated with allopurinol-induced cutaneous ADR, including SJS/TEN and DIHS/DRESS, in Caucasian and Asian patients, including the Japanese. These differences may, at least in part, be due to the differences in allele frequency in different ethnic populations. This article reviews the progress in pharmacogenomics, associated mainly with carbamazepine and allopurinol in different ethnic populations. Pharmacogenetic screening based on associations between adverse reactions and specific HLA alleles helps to avoid serious conditions associated with drug hypersensitivity.

Key words: adverse drug reaction, drug-induced hypersensitivity syndrome, pharmacogenetics, Stevens–Johnson syndrome, toxic epidermal necrolysis.

INTRODUCTION

Drug hypersensitivities develop in susceptible patients as adverse drug reactions (ADR) following exposure to certain drugs. Many of the ADR are thought to be immunologically mediated and are of major concern to clinicians, because severe hypersensitivity is life-threatening and cannot be predicted.

Toxic epidermal necrolysis (TEN) and Stevens–Johnson syndrome (SJS) are major cutaneous ADR characterized by destruction of the epidermis and mucosal epithelium, often with organ involvement. They are considered variants of the same disorder differentiated by the presence of skin separation and extent of the body surface area involved.[1,2] Although they are rare disorders, the mortality is as high as 1–5% for SJS and 20–30% for TEN.[3,4] Common drugs that cause SJS/TEN include allopurinol, anticonvulsants, antimicrobials, non-steroidal anti-inflammatory agents and aromatic sulphonamide, although many other drugs can be implicated in SJS/TEN.

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Drug-induced hypersensitivity syndrome (DIHS) or drug rash with eosinophilia and systemic symptoms (DRESS) are other severe cutaneous ADR. In patients with DIHS/DRESS, skin rash usually occurs more than 2 weeks after the initial administration of the drug, associated with fever, hepatitis and/or other internal organ involvement, lymphadenopathy and hematological abnormalities (leukocytosis, hypereosinophilia and atypical lymphocytosis). Reactivation of human herpesvirus (HHV), mainly HHV-6, and less frequently cytomegalovirus, has been described during the course of DIHS/DRESS.[5–8] These viral reactivations have been reported in association with recurrence of symptoms more than 2 weeks after the drug was discontinued.[7,8] Common drugs associated with DIHS/DRESS include aromatic amine anticonvulsants (carbamazepine, phenytoin and phenobarbital), allopurinol, minocycline, sulfa antimicrobials and aromatic sulfonamides.

Not only immunological but also genetic factors have recently been suggested to contribute to the pathogenesis of cutaneous ADR. This notion is supported by studies associating human leukocyte antigen (HLA) class I alleles with SJS/TEN induced by anticonvulsants. In addition to playing a role as a genetic marker for cutaneous ADR, the particular HLA molecule is also functionally involved in the pathogenesis of cutaneous ADR. The drug antigen (e.g. drug–peptide complex) is presented by the specific HLA molecule on the antigen-presenting cells and recognized by effector T cells through the T-cell receptor for HLA-restricted T-cell activation. HLA class I restricted $CD8^+$ T cells and HLA class II restricted $CD4^+$ T cells are thought to induce an immune response, including cutaneous ADR. In SJS/TEN, $CD8^+$ cytotoxic T cells in the skin lesions may play an important role in eliciting keratinocyte death.[9]

In 2004, a very strong association of HLA-B*1502 with carbamazepine-induced SJS/TEN was reported in southeast Asian patients[10–12] and patients of Asian ancestry living in Europe.[13] This was an epoch-making finding in the field of pharmacogenetics of cutaneous ADR. However, the association has not shown across different populations or ethnicities. Moderate associations between aromatic amine anticonvulsants and other HLA alleles have been proposed in different ethnic populations. In contrast, some studies showed an association between the HLA class I allele and allopurinol-induced ADR, including TEN/SJS and DIHS/DRESS, across different populations.[14,15]

This review is focused on the recent pharmacogenetic studies of cutaneous ADR, including TEN/SJS and DIHS/DRESS, mainly induced by carbamazepine and allopurinol, and hypersensitivity induced by antiretroviral drugs, and discusses future perspectives of pharmacogenomics in cutaneous ADR.

ALLELE ASSOCIATIONS WITH CUTANEOUS ADR INDUCED BY AROMATIC AMINE ANTICONVULSANTS

Recently, many studies on allele associations with cutaneous ADR induced by aromatic amine anticonvulsants have been reported in Asian and European populations. Current studies indicate that HLA-B*1502 is a marker for carbamazepine-induced SJS/TEN in southeast Asian populations, where the prevalence of HLA-B*1502 is relatively high.

ASSOCIATION BETWEEN HLA-B*1502 AND CARBAMAZEPINE-INDUCED SJS/TEN IN SOUTHEAST ASIAN AND EUROPEAN PATIENTS

In 2004, Chang *et al.*[10] reported a strong association between HLA-B*1502 and carbamazepine-induced SJS/TEN in Han-Chinese residing in Taiwan (Table 1). In this case–control study, 100% of 44 Han-Chinese SJS/TEN patients were HLA-B*1502 positive versus 3% of 101 tolerant patients and 8.6% in the general population ($P = 3.1 \times 10^{-27}$; odds ratio [OR] = 2505).[10] A follow-up study by Hung *et al.*[16] confirmed this association not only in Han-Chinese residing in Taiwan but also in those residing in Hong Kong and China and in Chinese descendents residing in the USA (98.3% of 60 patients, $P = 1.6 \times 10^{-41}$; OR = 1357). Further studies have confirmed the association between HLA-B*1502 and carbamazepine-induced SJS/TEN in Chinese, Thai and Indian populations.[11,12,17] Tassaneeyakul *et al.*[18] have performed a case–control study using 42 carbamazepine-induced SJS/TEN patients and 42 carbamazepine-tolerant controls in a Thai population. In their study, 37 SJS/TEN patients carried HLA-B*1502, thus suggesting a very strong association of HLA-B*1502 with

Table 1. Reported genetic biomarkers for anticonvulsants-induced cutaneous ADR

Causative drug	HLA-B	Race		Selectivity	References
Carbamazepine	*1502	Han Chinese (Taiwan)	SJS/TEN	59/60	16
		Han Chinese (Hong Kong)	SJS/TEN	4/4	11
		Asians in Europe	SJS/TEN	4/4	13
		Thai	SJS	37/42	18
		Indians	SJS	6/8	17
		Caucasians	SJS/TEN	0/8	13
		Japanese	SJS/TEN	0/15	22
		Han Chinese (Taiwan)	DIHS	0/13	16
		Caucasians	DIHS	0/56	29
	*1511	Japanese	SJS/TEN	4/15	22
Phenytoin	*1502	Han Chinese (Taiwan)	SJS/TEN	8/26	28
		Thai	SJS/TEN	4/4	18
Lamotrigine	*1502	Han Chinese (Taiwan)	SJS	2/6	28
Oxcarbazepine		Han Chinese (Taiwan)	SJS	3/3	28

ADR, adverse drug reactions; HLA, human leukocyte antigen; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis.

SJS/TEN ($P = 2.89 \times 10^{-12}$; OR = 54.76). In India, the same association was shown in six out of eight patients by Mehta *et al.*[17]

In contrast, this association has not been detected in Caucasian populations.[19] A European study performed by Lonjou *et al.*[19] included 12 carbamazepine-induced SJS/TEN patients, four HLA-B*1502-positive patients who had Asian ancestry and eight HLA-B*1502-negative Caucasian patients. These studies demonstrate that an association between HLA-B*1502 and carbamazepine-induced SJS/TEN is observed only in southeast Asian populations.

ALLELE ASSOCIATIONS WITH CARBAMAZEPINE-INDUCED SJS/TEN IN JAPANESE PATIENTS

In Japanese studies, none of the SJS/TEN patients receiving aromatic anti-epileptic drugs, including carbamazepine, carried HLA-B*1502 (Table 1).[20–23] Ueta *et al.* reported a case–control study on the relationships between HLA class I and II genetic polymorphisms with severe ocular complications using 71 Japanese drug-unspecified SJS/TEN patients and 101 Japanese controls. No HLA-B*1502 carriers were detected in cases or controls.[24] Instead, the investigators reported that HLA-A*0206 was associated with SJS/TEN with severe ocular complications ($P = 4 \times 10^{-5}$; OR = 4.1).[24]

Recently, our group detected four patients carrying HLA-B*1511 among 15 carbamazepine-induced SJS/TEN patients (26.7%). The allele frequency of HLA-B*1511 was significantly increased in the patients (13.3%) compared with that of the Japanese population (1%) ($P < 10^{-4}$; OR = 19.52).[22] These data suggest that HLA-B*1511, a member of the HLA-B75 group, as well as HLA-B*1502, are risk factors for carbamazepine-induced SJS/TEN in Japanese populations. Other major members of HLA-B75 are HLA-B*1508, HLA-B*1515 and HLA-B*1521. Interestingly, HLA-B*1508, HLA-B*1511 and HLA-B*1521 were detected in studies on SJS/TEN in Thailand and India.[12,17] These findings suggest that subfamilies belonging to the HLA-B75 serotype are involved in carbamazepine-induced SJS/TEN.

ALLELE FREQUENCIES OF INDIVIDUAL HLA-B75 (B*1502, B*1508, *1511, B*1515, B*1521) GENOTYPES

Allele frequencies of individual HLA genotypes in worldwide populations are shown at www.allelefrequencies.net (Table 2).[25] The prevalence of HLA-B*1502 is relatively high in southern Chinese and southeast Asian populations where HLA-B*1502 is in fact a marker for carbamazepine-induced SJS/TEN.[22] In contrast, the prevalence of HLA-B*1502 is very low in Caucasian and Japanese populations. This suggests that one reason for not detecting HLA-B*1502 in carbamazepine-induced SJS/TEN in Japanese and Caucasian patients is the low allele frequency. In addition, the extremely low allele frequencies of HLA-B75 subfamilies in Caucasians

Table 2. Population allele frequencies of major subfamilies of serotype B75

	Population allele frequencies reported in www.allelefrequencies.net website[†]				
Ethnic group	HLA-B*1502	HLA-B*1515	HLA-B*1521	HLA-B*1508	HLA-B*1511
Japanese	0.001				0.004–0.008[‡]
Koreans	0.002	0	0	0	0.02
Han Chinese	0.019–0.124	0.01	0.000–0.002	0.005–0.015	0.000–0.017[§]
Thai	0.061–0.085		0.007–0.010	0.01	0.01
Indians	0.000–0.060			0.005–0.033	
Caucasians	0	0	0	0.000–0.004	0.000–0.003

[†]New Allele Frequency Database: www.allelefrequencies.net/ (Middleton *et al.*).[25] [‡]The frequency of 0.1 was reported by Tanaka *et al.*[45] [§]Higher value than 0.038 in Han Chinese in Beijing was recently reported by Yang *et al.*[46] This table was partly quoted from Kaniwa *et al.*[22] HLA, human leukocyte antigen.

may be a reason for detecting no HLA-B75 subfamilies, including HLA-B*1511, in the Caucasian patients with carbamazepine-induced SJS/TEN.

HLA-B ASSOCIATION IN OTHER AROMATIC AMINE ANTICONVULSANT-INDUCED SJS/TEN

Aromatic amine anticonvulsants such as carbamazepine, phenytoin, phenobarbital, oxcarbazepine and lamotrigine are metabolized to arene oxide metabolites. Clinical cross-reactivity among aromatic amine anticonvulsants is observed with high frequency.[26,27] Small case studies in Thailand (four cases phenytoin induced) and Hong Kong (single cases of phenytoin and lamotrigine induced) showed the presence of HLA-B*1502 in all SJS patients.[11,12] In a current case–control association study in a Taiwanese population, the association between HLA-B*1502 and phenytoin-, lamotrigine- and oxcarbazepine-induced SJS/TEN was observed in 30.8% of 26 patients ($P = 4.1 \times 10^{-3}$; OR = 5.1), 33% of six patients ($P = 1.3 \times 10^{-1}$; odds ratio = 5.1) and 100% of three patients ($P = 8.4 \times 10^{-4}$; OR = 80.7), respectively.[28] These results indicate that aromatic anticonvulsants share a common risk allele, HLA-B*1502, presumably by similar antigen recognition, although the association is highest with carbamazepine. Other genetic factors may also contribute to the pathomechanism of the disease. Thus, HLA-B*1301, Cw*0801 and DRB1*1602 also showed an association with phenytoine-SJS/TEN in the same study ($P = 0.0128$–0.0281; OR = 3.0–4.3).[28]

In Europe, where the allele frequency of HLA-B*1502 is extremely low, a rare allele, HLA-B*38, showed a weaker association ($P < 2 \times 10^{-2}$; OR = 6.8) with SJS/TEN in a limited number of patients treated with lamotrigine.[15]

ALLELE ASSOCIATIONS WITH DIHS/DRESS AND MACULOPAPULAR ERUPTION INDUCED BY AROMATIC AMINE ANTICONVULSANTS

In addition to SJS/TEN, carbamazepine also induces other types of cutaneous ADR, including maculopapular eruption (MPE) and DIHS/DRESS. The association between HLA-B*1502 and carbamazepine-induced MPE was not detected in Han-Chinese populations in Taiwan and Hong Kong or in the Thai population.[11,12,16] Studies in 18 Han-Chinese patients residing in Taiwan and 56 Caucasian patients showed that carbamazepine-induced DIHS/DRESS was not associated with HLA-B*1502.[16,29] These data suggest that the association between HLA-B*1502 and carbamazepine-induced cutaneous ADR is specific to SJS/TEN.

Kano *et al.*[30] showed that four out of 13 Japanese patients (30.8%) with DIHS/DRESS – all associated with HHV-6 reactivation – induced by aromatic amine anticonvulsants (carbamazepine, eight; phenobarbital, two; phenytoin, one) had HLA-B*1301 (allele frequency 15.4%). This allele frequency of HLA-B*1301 was much higher than that reported for the Japanese population (1.3%),[31] although the difference was not statistically significant after correction for multiple comparisons. They supposed that the effect of certain HLA-B alleles on the virus reactivation contributed, in part, to the HLA-B allele association with DIHS/DRESS.

Recently, we found a significant association between carbamazepine-induced cutaneous ADR

and HLA-A*3101 in 22 Japanese patients, including MPE, erythema multiforme, erythroderma, DIHS, SJS and other types. Eleven patients (50%), including two SJS patients and others, carried HLA-A*3101, and the allele frequency was much higher in the patients (25%) than that reported for the Japanese population (7.1%) ($P = 4 \times 10^{-4}$; OR = 4.33).[23] Another study involving carbamazepine-induced MPE in 18 Han-Chinese also suggested the association with HLA-A*3101 ($P = 2.2 \times 10$; OR = 17.5).[16] The sample sizes of these studies were small, so further study on a large sample size is needed to clarify whether or not HLA-A*3101 is a risk allele.

ASSOCIATION BETWEEN HLA-B*5801 AND ALLOPURINOL-INDUCED CUTANEOUS ADR

Allopurinol is a xanthine oxidase inhibitor used to treat gout and hyperuricemia (Table 3). A case–control study in a Han-Chinese population showed an extremely strong association between HLA-B*5801 and allopurinol-induced SJS/TEN or DIHS/DRESS.[14] In this study, all 51 patients (100%) with allopurinol-induced SJS/TEN or DIHS/DRESS carried HLA-B*5801, compared with only 20 out of 135 (15%) allopurinol-tolerant patients and 19 out of 93 (20%) population controls ($P < 10^{-6}$; OR = 580). Regarding the association in other southeast Asian populations, a similar strong association between HLA-B*5801 and allopurinol-induced SJS/TEN was shown in a case–control study in a Thai population.[32]

The association of HLA-B*5801 with allopurinol-induced SJS/TEN was observed in a European study as well ($P < 10^{-8}$; OR = 80).[15] The carrier frequency was 55% in 27 European patients. One of the reasons for the lower carrier frequency seems to be the lower allele frequency of HLA-B*5801 (1–6%) in the European population than in southeast Asian populations, although HLA-B*5801 is more broadly distributed than HLA-B*1502.

In the Japanese population, the allele frequency of HLA-B*5801 is less than 1%.[20] We have reported earlier that four out of 10 Japanese patients (40%) with allopurinol-induced SJS/TEN carried HLA-B*5801.[20] A moderate but statistically significant association ($P < 10^{-4}$, OR = ~40) between HLA-B*5801 and allopurinol-induced SJS/TEN was detected in that study. Our recent data have shown that 10 out of 18 Japanese patients (55.6%) with allopurinol-induced SJS/TEN carried HLA-B*5801 (M. Tohkin, unpubl. data, 2010). Dainichi *et al.*[33] also detected three HLA-B*5801 carriers in all three allopurinol-treated Japanese patients diagnosed with SJS, DIHS and TEN, respectively. Although the sample size in our study was not sufficient to estimate the accurate carrier frequency in Japanese patients, it showed a possible association between HLA-B*5801 and allopurinol-induced SJS/TEN in Japan.

These studies lead to the conclusion that HLA-B*5801 is a potential genetic biomarker for allopurinol-associated SJS/TEN across different populations or ethnicities, although there is less information regarding the association in other populations than the Japanese.

ALLELE ASSOCIATION WITH CUTANEOUS ADR INDUCED BY ANTIRETROVIRAL DRUGS

HIV patients treated with antiretroviral drugs show a high frequency of cutaneous ADR, including SJS/TEN and hypersensitivity syndrome (Table 4). The hypersensitivity syndrome is associated with fever,

Table 3. Reported genetic biomarkers for allopurinol-induced cutaneous ADR

HLA-B	Race	ADR	Selectivity	References
*5801	Han Chinese (Taiwan)	SJS/TEN or DIHS/DRESS	51/51	14
	Thai	SJS/TEN	27/27	32
	Caucasians	SJS/TEN	15/27	15
	Japanese	SJS/TEN/DIHS	3/3	33
	Japanese	SJS/TEN	4/10	20

ADR, adverse drug reactions; DIHS, drug-induced hypersensitivity syndrome; DRESS, drug rash with eosinophilia and systemic symptoms; HLA, human leukocyte antigen; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis.

Table 4. Reported genetic biomarkers for cutaneous ADR induced by antiretroviral drugs

Causative drug	HLA	Race	ADR	Selectivity	References
Abacavir	B*5701	Caucasians	Hypersensitivity	57/130	40
			Hypersensitivity (patch test+)	42/42	40
		Black	Hypersensitivity	10/69	40
			Hypersensitivity (patch test+)	5/5	40
		Japanese	Hypersensitivity	0/7	38
Nevirapine	B*3505	Thai	Hypersensitivity	25/143	42
	Cw8	Japanese	Hypersensitivity	5/12	44
	Cw8	Sardinian	Hypersensitivity	6/3	43
	B14	Sardinian	Hypersensitivity	6/13	43

ADR, adverse drug reactions; HLA, human leukocyte antigen.

rash and internal organ involvement (gastrointestinal symptoms in abacavir-treated patients and hepatitis in nevirapine-treated patients).[34]

ABACAVIR

Abacavir is a guanosine analog that belongs to the family of nucleoside reverse transcriptase inhibitors used for treatment of HIV infection. Hypersensitivity to abacavir occurs in approximately 5–8% of patients within 1–6 weeks of the initial dose.[35] The initial association between abacavir-induced hypersensitivity and HLA-B*5701 was reported in Australian and British populations.[36,37] However, abacavir-induced hypersensitivity is present at a high frequency only in Caucasians and at a very low frequency in Asian and black populations.[38,39] In fact, the allele frequency of HLA-B*5701 is approximately 8% in Caucasians, but lower in Asian and African populations.[36,37] To examine the universality of the sensitivity and specificity of HLA-B*5701 association with abacavir hypersensitivity across ethnicities, the Study of Hypersensitivity to Abacavir and Pharmacogenetic Evaluation (SHAPE) was performed. It was a case–control study that enrolled both white and black patients in the USA.[40] This study showed that 100% of both white and black patch test-positive patients carried HLA-B*5701, suggesting a predictive value of HLA-B*5701 for abacavir-induced hypersensitivity across ethnicities. This study demonstrated the clinical utility of testing for HLA-B*5701 prior to prescription of abacavir.

Although all current studies show the requirement for HLA-B*5701 presence for development of abacavir-induced hypersensitivity syndrome, 45% of patients who carry HLA-B*5701 do not develop the hypersensitivity syndrome.[41] Therefore, it is likely that HLA-B*5701 is necessary but not sufficient for development of abacavir-induced hypersensitivity syndrome.

NEVIRAPINE

Nevirapine is another antiretroviral agent that is a potent non-nucleoside reverse transcriptase inhibitor. Nevirapine often causes cutaneous ADR with a frequency of approximately 5% for hypersensitivity syndrome and 0.3% or less for SJS/TEN.[34] A recent case–control study in Thailand showed a high frequency of HLA-B*3505 (17.5%) in patients with nevirapine-induced hypersensitivity syndrome.[42] Because HLA-B*3505 is carried by less than 1% of the Thai population, a strong association between HLA-B*3505 and nevirapine-induced hypersensitivity syndrome is suggested. HLA-Cw8 and HLA-B*1402 associations with nevirapine-induced hypersensitivity were also reported in a Sardinian population,[43] and a HLA-Cw8 association was noted in a Japanese population.[44] To date, no specific HLA association has been described in nevirapine-induced SJS/TEN.

FUTURE PERSPECTIVE OF PHARMACOGENOMICS IN CUTANEOUS ADR

Elucidation of associations between HLA alleles and drug hypersensitivity will make it possible to predict immunologically mediated drug reactions and prevent them in the future. As a result of current studies, the strong associations between HLA-B*1502 and

carbamazepine-induced SJS/TEN in patients of Asian ancestry, and between HLA-B*5701 and abacavir hypersensitivity, have been included in the labels on these drugs, and screening for these alleles is recommended by the US Food and Drug Administration (FDA) prior to initiating therapy with these drugs. The FDA has also updated the genetic information on other drug labels and now recommends genetic testing for more than 10 drugs. In order to perform a widespread genetic screening for these drugs, technology advancement is needed to decrease the cost of the screening. It is to be hoped that pharmacogenetic testing kits will be available in the near future for prevention of severe reactions such as SJS/TEN and hypersensitivity syndrome. In addition, the ability to predict the propensity of drugs to cause ADR will make pharmacogenomic screening an important tool in new drug development in the future.

Although strong associations have been shown between HLA alleles and some types of cutaneous ADR, there has been no definitive proof or data published concerning the functions of the implicated HLA alleles. HLA-restricted T-cell activation is needed for induction of immunological reactions and, in addition, there is a possibility that some HLA proteins have higher binding affinity than others toward a drug or drug metabolite through covalent or non-covalent mechanisms. On the other hand, a protecting effect of HLA has been suggested. Alfirevic *et al.*[29] reported a potential protecting effect of HLA-B*0702 against carbamazepine-induced severe cutaneous adverse reactions in Caucasian patients. Functional studies together with genomic approaches are required for further progress in understanding the pathogenesis of ADR.

Many questions are still unresolved. For instance, it is still unclear what the genetic difference is between the patients who develop severe reactions such as SJS/TEN and milder skin reactions, and between the patients who develop severe skin reactions and those who have only internal organ involvement with the same drug. In order to elucidate the pathogenesis of these diseases, definite case–control studies will be needed.

For further development of pharmacogenomics, collaboration between different research groups is needed to collect larger numbers of biological samples from ADR patients, particularly from those with rare ADR such as SJS/TEN. This association is also needed across ethnicities, based on consistent definitions of diseases.

CONCLUSIONS

Studies presented in this review show the tremendous progress in the area of pharmacogenetics of cutaneous ADR in recent years. It is likely that further progress will be made in this field by the continuous development of genetic technologies and the international well-defined sampling of the patients. This could result in the reduction of serious cutaneous ADR by screening prior to initiating drug therapies. Further studies, such as confirmatory haplotype-mapping, are required to definitively identify the susceptibility region responsible for the hypersensitivity.

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CED
Clinical and Experimental Dermatology

CPD

1 Utility of patch testing for patients with drug eruption

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Summary

Background. Patch testing is less dangerous than oral provocation testing for identification of the causative drug for patients with drug eruption; however, its usefulness for such identification is controversial.

Aim. To clarify the rates of positive patch testing for patients with drug eruption, classified by causative drugs and clinical features.

Methods. We analysed results during the period 1990–2010 for 444 patients (151 men, 293 women; mean ± SD age 49.9 ± 18.6 years) who were tested for drug eruption. In the patient group, there were 309 (69.1%) with maculopapular eruption and 31 (6.9%) with severe drug eruption. The test materials were applied to the back and left for 2 days under occlusion, then results were assessed by the International Contact Dermatitis Research Group (ICDRG) scoring system 3 days after application. Reactions of + to +++ were regarded as positive.

Results. Of the 444 patients, 100 (22.4%) had a positive patch test result to a suspected drug. Positive rates were 23.6% and 20.0% for maculopapular eruption and fixed drug eruption, respectively. The class of materials to which most patients reacted positively was contrast medium (n = 53; 41.1%), followed by drugs acting on the central nervous system (n = 18; 28.6%). In the latter group, 16 of the 18 patients were positive to antiepileptics.

Conclusions. Positive rates depend on the causative drug rather than the clinical features of the drug eruption. Patch testing is useful when contrast medium or antiepileptics are suspected to be the causative drugs. However, standardization of patch test materials and method of reading is needed, as well as guidelines regarding when testing should be performed. Although patch testing for drug eruption has significant potential, it requires further validation.

Introduction

The oral provocation test is a reliable method to identify causative drugs for patients with drug eruption.[1] However, it may be dangerous in some serious drug eruptions, such as Stevens–Johnson syndrome (SJS), toxic epidermal necrolysis (TEN) or drug-induced hypersensitivity syndrome (DIHS), as these conditions have a guarded prognosis.[2,3] Patch testing is less dangerous than the oral challenge test, but its usefulness for drug eruption is controversial.[4] To determine the utility of patch testing for patients with drug eruption, we analysed the results of patch testing for patients with drug eruption over a 20-year period.

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Methods

Patients

We analysed the results for patients patch-tested at the Department of Dermatology, Showa University Hospital, during the period April 1990 to March

2010. In total, 444 patients (151 male, 293 female; mean ± SD age 49.9 ± 18.6 years, range 2–93) suspected of having drug eruption were tested (Table 1). Table 2 shows the frequency of the clinical types of drug eruptions. Of the 444 patients, 309 (69.6%) had maculopapular eruption. Because this type of eruption is often difficult to distinguish from viral infection, patients were identified on the basis of their medical history and the clinical course pf the disease, as follows: (i) rash occurring up to 4 weeks after drug administration, and (ii) improvement of lesions after the suspect medication was stopped. There were 55 (12.4%) and 37 (8.3%) patients, respectively with fixed drug eruption (FDE) and erythema multiforme (EM). A further 31 patients (7.0%) were diagnosed as having severe drug eruptions (SJS, TEN or DIHS). Table 3 shows the number of tested patients for each class of drug: 129 were tested with contrast media, 126 with antibacterial agents and 101 with nonsteroidal anti-inflammatory drugs (NSAIDs).

Patch testing

Patch-testing materials (Manufacturing Laboratory, Showa University Hospital Pharmacy) were applied to the back of each participant, and left for 2 days under occlusion. Two types of patch-test unit were used: vinyl plaster (Mini-plaster or Patch Tester Torii; Torii Pharmaceutical Co, Ltd, Tokyo, Japan) for water-based materials and Finn Chamber® (Smart-Practice Co. Ltd, Yokohama, Japan) for petrolatum-based materials. In 33 of the 55 patients with FDE, patch tests were performed both on normal skin and on the affected site, while the other 22 were tested on the back because of difficulty applying the drug to the affected sites (e.g. lip, genital region). In four cases of suspected photosensitivity, photopatch testing was preformed: two materials were applied to the back, then one was removed after 24 h and the area irradiated with half the minimal erythema dose of ultraviolet (UV)A/UVB (Dermaray UV; Eisai Co. Ltd, Tokyo, Japan).

Table 1 Patient characteristics.

Group	*n*	Age, years
All	444	49.9 ± 18.6*
Men	151	50.9 ± 18.4
Women	293	49.5 ± 18.8

*Data are mean ± SD.

All the tests were performed between 2 weeks and 4 months after onset of eruption. Results were assessed using the International Contact Dermatitis Research Group (ICDRG) scoring system 3 days after application.[5] Reactions of + to +++ (Ph+ to Ph+++ for photopatch testing) were regarded as positive. Any results that were difficult to assess because of technical limitations were excluded.

Table 2 Incidence and positive rate related to clinical features/condition.

Clinical features/condition	Patients, *n*	Positive reaction, *n*	Positive rate, %
Maculopapular eruption	305	73	23.9
FDE	55	11	20
EM	37	3	8.1
Photosensitivity	4	2	50
AGEP	3	0	0
Severe reactions			
SJS	7	1	14.3
TEN	8	0	0
DIHS	16	9	56.3
Others	9	1	11.1
Total	444	100	22.5

AGEP, acute generalized exanthematous pustulosis; DIHS, drug-induced hypersensitivity syndrome; EM, erythema multiforme; FDE, fixed drug eruption; SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis.

Table 3 Incidence and positive rate related to drugs.

Drug	Patients, *n*	Positive reaction, *n*	Positive rate,%
Contrast agent	129	53	41.1
Drugs acting on the CNS	63	18	28.6
Antiepileptics	39	16	41.0
Nonsteroidal anti-inflammatory drugs	101	11	10.9
Antibacterial and antifungal agents	126	9	7.1
Drugs acting on the respiratory system	51	3	5.9
Cold remedies	65	2	3.1
Traditional Chinese medicine	14	2	14.3
Muscle relaxants	4	2	50.0
Drugs affecting metabolism and GI function	74	1	1.4
Metal antagonists	4	1	25.0
Drugs acting on the cardiovascular system	48	0	0
Drugs acting on the immune response	34	0	0
Vitamins	10	0	0
Others	20	0	0

CNS, central nervous system; GI, gastrointestinal.

Results

Of the 444 patients suspected of having drug eruption, 100 (22.4%; 39 men, 61 women; age 51.2 ± 17.1 years, range 17 to 86) had positive patch-test results. Positive rates in cases of maculopapular eruption, FDE and EM were 23.9%, 20.0% and 8.1%, respectively (Table 2). Regarding the severe drug eruptions, positive rates in cases of SJS, TEN and DIHS were 14.3%, 0% and 56.3%, respectively (Table 2). Contrast medium was the most common source of positive patch test (n = 53; 41.1%), and the second most common (n = 18; 28.6%) was the class of drugs acting on the central nervous system (CNS) (Table 3). In the latter group, 16 of 18 (41.0%) reacted to antiepileptics and 12 of the 16 had positive reactions to carbamazepine (Table 3). Of 101 patients tested with NSAIDs, 11 (10.9%) had a positive reaction (Table 3), while of 126 patients tested with antibacterial or antifungal agents, 9 (7.1%) tested postive (Table 3). Rates of positive reactions to drugs acting on the respiratory system, cold remedies, and traditional Chinese medicines were 5.9%, 3.1% and 14.3%, respectively (Table 3). No patients reacted to drugs acting on the cardiovascular system or the immune response, or to any vitamins (Table 3).

Discussion

In this study, the positive response rate to suspected drugs in our cohort of 444 patients was 22.4% but positive rates in patients with photosensitivity or DIHS were > 50% (Table 2). The drug to which most patients reacted was contrast medium (Table 3). Numerous drug eruptions were reported in Japan shortly after the introduction of nonionic iodinated contrast media,[6] with the main clinical feature being papulomacular eruption, partticularly oedematous erythema and papules, mainly on the trunk.[6] We suggest two reasons for the numerous cases of drug eruption caused by contrast media in Japan.[6] First, deficiency of aldehyde dehydrogenase (ALDH), a migrating isoenzyme of liver acetaldehyde dehydrogenase, which occurs in around 50% of Japanese (i.e. they have a low Michaelis constant and K_m for acetaldehyde) may play a role.[7,8] Second, until 2000, the rate of annual exposure to contrast medium was higher, as it included pretesting performed with a small amount of contrast medium, as it was thought that this pretesting might predict those patients likely to have adverse reactions.[6,9] The ddiscontinuation of this pretesting procedure might be the reasons for the decrease in the positive rates and numbers of patients affected: 43.4% (46/106) during the period April 1990 to March 2000, and 30.4% (7/23) in the period April 2000 to March 2010.

Drugs acting on the CNS made up the second largest group of positive tests, with 18 patients reacting to them (Table 3): 16 reacted to antiepileptics and 12 to carbamazepine.Regarding DIHS, 9 of 16 tested patients with DHHS (56.3%) had positive results (Table 2); this high positive rate was not due to DIHS itself, but rather to the causative drugs, as 8 of the 9 patients reacted to carbamazepine. It is well known that carbamazepine results in high positive rates in patch testing.[10] Indeed, a limitation of patch testing for drug eruption lies in differentiating between systemic and cutaneous metabolism of drugs.[11] We speculate that this difference might be less for carbamazepine than for other drugs because this drug has a uniform distribution through the body,[12] and cases of flare-up phenomena during patch testing with carbamazepine have been reported[13]. Our data therefore suggest that the positive rates may depend on the causative drug rather than on the clinical features of the drug eruptions. However, the positive rate for FDE might have been affected by the negative reactions occurring in 22 patients who had the materials to their back, as positive results are more likely when materials are applied to the involved skin.

Patch testing is much safer than oral challenge or intracutaneous test for the identification of a causative drug in patients with drug eruption.[14] Our results suggest that patch-testing can be useful when contrast medium or antiepileptic is suspected as the causative drug, and can be relevant to patient management.[11,15] However, the general opinion of patch testing for patients with drug eruption is not high,[16] and negative reactions are not useful.[17] To perform patch testing for drug eruptions, patient consent must be gained after they are informed as to the limitations of the testing, as described above and the influence of the testing on their social life, e.g. avoidance of showers, sunbathing and exercise. Although some guidelines have been defined,[11,18] we consider that three standardized metrics are needed to overcome the issues described above if patch testing is t be used for drug eruptions.

First, the actual treatment drugs should be used as patch test allergens. There is a limited number of drugs that are commercially available for patch testing,[19–21] thus most are formulated by each local facility, leading to difficulties in comparing data between different facilities. Each drug also has an optimal patch test concentration that is not irritant, and optimal

Table 4 Patch test concentration and vehicles of main causative drugs.

Classification	Drug	Concentration, %	Vehicle
Contrast medium	Iohexol	As supplied (water)	
	Iopamidol	As supplied (water)	
	Iomeprol	As supplied (water)	
Drugs acting on the CNS	Carbamazepine	1	Pet.
	Phenytoin	1	Pet.
	Phenobarbital	1	Pet.
NSAIDs	Diclofenac sodium	10	Pet.
	Mefenamic acid	10	Pet.
	Acetoaminophen	10	Pet.
	Piroxicam	1	Pet
Antibacterial agents	Amoxicillin trihydrate	10	Pet.
	Ampicillin hydrate	10	Pet.
	Minocycline hydrochloride	10	Pet.
	Clarithromycin	10	Pet.
	Cefaclor	10	Pet.
	Cefalexin	10	Pet.
Others	Dihydrocodeine phosphate	10	Pet.

CNS, central nervous system, NSAID, nonsteroidal anti-inflammatory drug, pet., petrolatum.

vehicle(s). Table 4 shows the patch-test concentrations and vehicles of the main causative drugs used in this study. There is a limited number of commercially available drugs for patch testing,[19–21] thus most are formulated by each local facility, leading to difficulties in comparing data between different facilities. In addition, where possible, patch testing should also be carried out using the individual ingredients of the medicines,[22] as these ingredients may also be used in other products.

Second, assessment of patch-test reactions for drug eruption needs to be standardized. The ICDRG scoring system is an established method of identifying allergens in cases of allergic contact dermatitis (ACD), but it can be difficult to interpret a reaction of '?+'in drug eruptions. In our study, 13 of 14 patients (92.9% with '?+' reactions to contrast media had a positive reaction to intracutaneous testing). In addition, although it is recommended that for ACD, readings should be performed on day 2, day 3 or 4, and day 7 in ACD, there are no similar data for drug allergy.

Third, it is unclear when patch testing should be performed immediately after improvement of the rash or several weeks later. Bruyzeel and Maibach[4] suggested the latter is better than the former, but Grandhe *et al.*[23] reported a longer interval occurred between the rash and evaluation in patients with negative patch-test reactions than in patients with positive patch-test reactions.

However, each clinical condition might have an optimal time for patch testing. Kano *et al.*[24] suggested that the lymphocyte transformation test should be performed within 1 week after the onset of rash in patients with maculopapular drug eruption and SJS/TEN, but should be delayed until 5–8 weeks after rash onset in DIHS. Similar data for patch testing will be necessary to increase its usefulness.

The fact that the oral challenge test is rarely used for drug verification demonstrates that this test is also not well standardized. It is also necessary to clarify when intradermal testing should be carried out if patch testing is negative.[2] Thus, further studies are necessary, both for patch testing and for other tests to detect the causative drug in patients with drug eruptions.

Conclusion

in terms of increased refinement of the evidence-based diagnosis of clinical relevance, patch testing for drug eruption has significant potential.[25] However, further validation is necessary to overcome the limitations of the test and to clarify the optimal conditions for its performance.

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CPD questions

Learning objective

To detail how to use patch testing to identify the causative drug for in patients with drug eruption.

Question 1

Which type of drug-induced eruption is the most common?
a) Maculopapular eruption.
b) Fixed drug eruption.
c) Erythema multiforme-type.
d) Photosensitivity.
e) Stevens–Johnson syndrome.

Question 2

Which of the following factors has the greatest effect on positive rates of drug eruption?
a) Patient age.
b) Clinical features of the drug eruption.
c) Clinical course of the drug eruption.
d) Severity of the drug eruption.
e) Causative drug.

Question 3

Which of the following is the most useful for identification of the cause of drug eruption by patch testing?
a) Antibacterial and antifungal agents.
b) Cold remedies.
c) Contrast medium.
d) Drugs acting on the cardiovascular system.
e) Drugs affecting metabolism and gastrointestinal function.

Question 4

Which of the following is the best vehicle for carbamazepine for patch testing?
a) 0.1% pet.
b) 0.1% water.
c) 1% pet.
d) 1% water.
e) 10% pet.

Question 5

As a method to identify the causative drug for patients with drug eruption, how should patch testing be regarded?

a) Not useful.
b) Useful only when antiepileptics are suspected.
c) Useful when clinical feature is not severe.
d) It has significant potential, but further validation is necessary.
e) Perfect.

Instructions for answering questions

This learning activity is freely available online at http://www.wileyhealthlearning.com/ced.

Users are encouraged to

- Read the article in print or online, paying particular attention to the learning points and any author conflict of interest disclosures.
- Reflect on the article.
- Register or login online at www.wileyhealthlearning.com/ced and answer the CPD questions.
- Complete the required evaluation component of the activity.

Once the test is passed, you will receive a certificate and the learning activity can be added to your RCP CPD diary as a self-certified entry.

Author Query Form

Journal: CED

Article: 12239

Dear Author,
During the copy-editing of your paper, the following queries arose. Please respond to these by marking up your proofs with the necessary changes/additions. Please write your answers on the query sheet if there is insufficient space on the page proofs. Please write clearly and follow the conventions shown on the attached corrections sheet. If returning the proof by fax do not write too close to the paper's edge. Please remember that illegible mark-ups may delay publication.

Many thanks for your assistance.

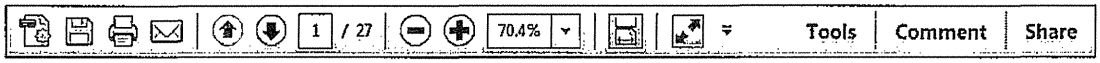
Query reference	Query	Remarks
1	**AUTHOR: The term "send to authors" has been deleted in this article. Please check.**	✓
2	**AUTHOR: 'longer interval occurred' correct? Please check.**	✓
3	**AUTHOR: Please provide the accessed date for references [19–21].**	✓

WILEY-BLACKWELL

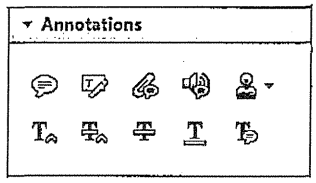
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 8.0 or above). (Note that this document uses screenshots from Adobe Reader X)
The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

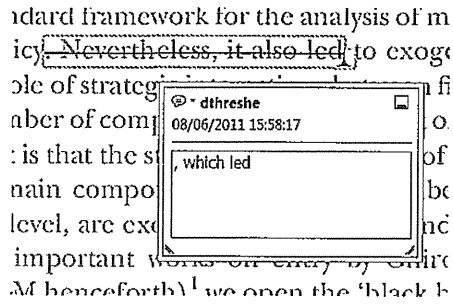


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

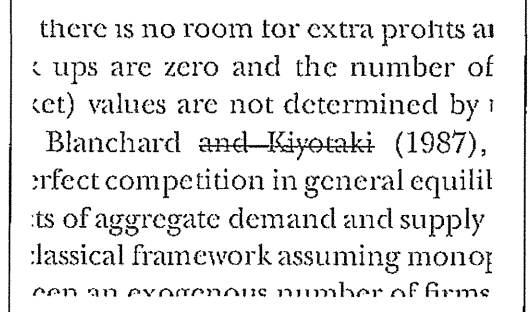


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

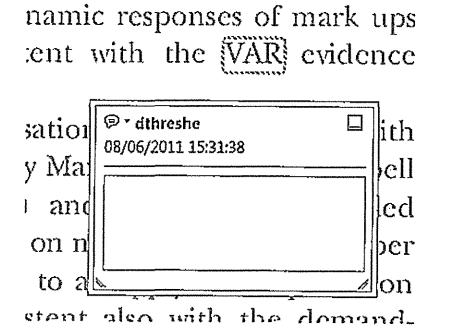


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

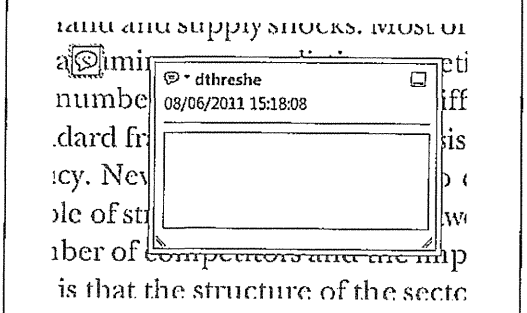


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



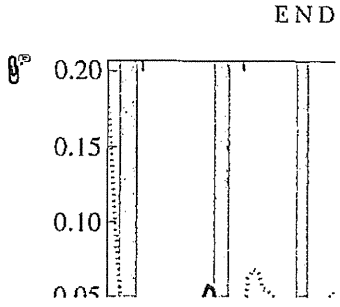
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

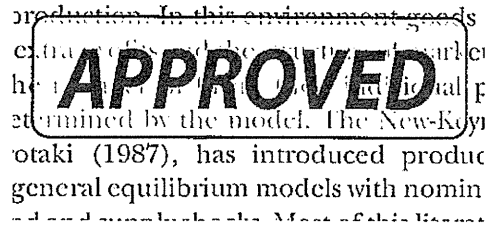


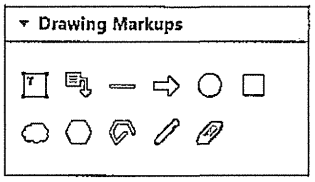
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).



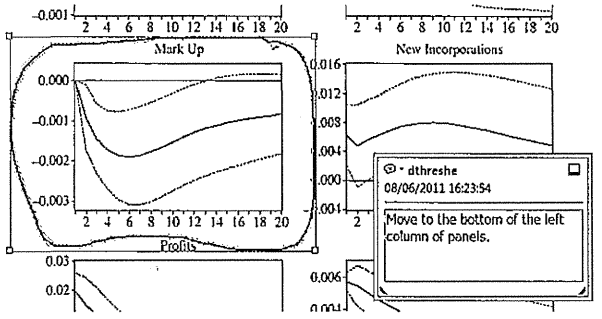


7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

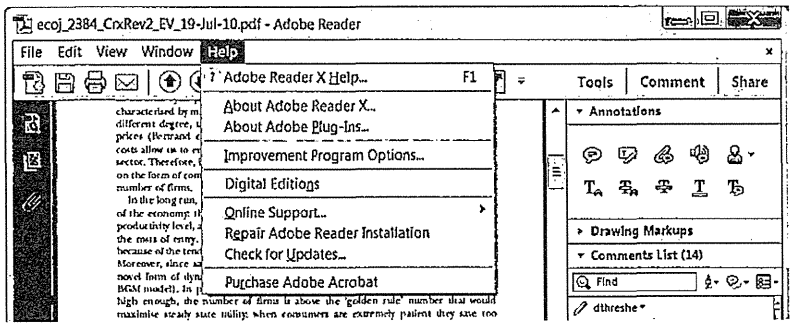
Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



For further information on how to annotate proofs, click on the Help menu to reveal a list of further options:



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ORIGINAL ARTICLE

Dermatological side-effects of telaprevir-based triple therapy for chronic hepatitis C in phase III trials in Japan

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ABSTRACT

Telaprevir-based triple therapy is highly effective for chronic hepatitis C. However, concern has been expressed over the high frequency and severity of its dermatological side-effects compared with those associated with peginterferon (PEG-IFN) and ribavirin (RBV) therapy. Thus, here, we evaluated the dermatological adverse reactions of telaprevir-based triple therapy in Japanese multicenter phase III clinical trials in an attempt to characterize the dermatological side-effects and establish appropriate management plans. In these trials, 126 treatment-naïve patients and 141 treatment-failure patients were administrated telaprevir, PEG-IFN-α-2b and RBV for 12 weeks followed by PEG-IFN-α-2b and RBV for another 12 weeks (T12/PR24 group), and 63 treatment-naïve patients were administrated PEG-IFN-α-2b and RBV for 48 weeks (PR48 group). Dermatological adverse reactions developed in over 80% patients in both groups, and most of them were grade 1 or 2. In the T12/PR24 group, there were more grade 2 or grade 3 events, and the time to onset was earlier than that in the PR48 group. Most reactions could be managed with topical corticosteroids and oral antihistamines, and the rates of discontinuation due to dermatological reactions were not high even in the T12/PR24 group. In the T12/PR24 group, however, two cases of Stevens–Johnson syndrome and one case of drug rash with eosinophilia and systemic symptoms, which corresponds to drug-induced hypersensitivity syndrome in Japan, were reported. For appropriate treatments of individual dermatological adverse reactions, the judgment of discontinuation of antiviral drugs and treatment based on the severity are extremely important in this triple therapy.

Key words: chronic hepatitis C, dermatological adverse reaction, drug rash with eosinophilia and systemic symptoms, Stevens–Johnson syndrome, telaprevir.

INTRODUCTION

Telaprevir, a novel direct-acting antiviral, inhibits the NS3-4A serine protease of hepatitis C virus (HCV) and suppresses HCV replication.[1] Triple therapy of telaprevir, peginterferon (PEG-IFN) and ribavirin (RBV) has proved to be more effective for treating chronic hepatitis C (CHC) compared to PEG-IFN and RBV combination therapy.[2–7]

In Japan, three phase III trials for telaprevir-based triple therapy were performed on treatment-naïve (TN) patients (patients who had never been treated with IFN agents), relapsers (patients who had undetectable HCV RNA during previous therapy for CHC), and non-responders (patients who never achieved undetectable HCV RNA during previous therapy for CHC). In these trials, the sustained virological response (SVR) rates of patients were as follows: TN patients, 73.0% in T12/PR24 group, 49.2% in PR48 group; relapsers, 88.1%; and non-responders, 34.4%.[6,7] The established duration of telaprevir-based triple therapy is 24 weeks, which is half of that for PEG-IFN and RBV therapy. Therefore, the higher efficacy of triple therapy achieved over a shorter period make it markedly superior to PEG-IFN and RBV therapy.

Dermatological side-effects were also observed in Japanese trials of telaprevir monotherapy that lasted for 12 or 24 weeks. The severity of the side-effects was mild to moderate, and two patients discontinued telaprevir due to development of skin disorders (pruritic rash or herpes zoster).[8,9]

Peginterferon and ribavirin combination therapy has also been known to cause cutaneous adverse reactions.[10,11] Even with IFN monotherapy, dermatological reactions were observed

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