Dilution, and so forth. All of these tests emphasize *in vitro* toxicity of either substrate materials or extract solutions to cultured cells. Cellular damage is observed and graded. Two available versions are included in Practice F 813 and Test Method F 895. An application-specific method is included in Practice F 1027. An HIMA/PMA guideline is available from the FDA for a discussion and references on other versions of this test.

6.2.3 Since the biological reaction to particles generated during function may differ from the reaction to soluble products, *in vitro* testing of macrophage/monocyte interaction with representative particles (Practice F 1903) may be considered.

6.3 *Sensitization Test*—The guinea pig maximization test (Practice F 720) is a procedure whereby the material (or extracts thereof) is mixed with Freund's complete adjuvant and administered to the test animals during a 2-week induction period. After 2 weeks' rest, the guinea pigs are challenged with the test substance and the skin graded for allergic reaction after 24 hours. Other test methods such as the guinea pig split adjuvant and closed patch test (Practice F 2147) or the repeated dermal patch may also be used. The mouse local lymph node assay (Practice F 2148) should be considered as an alternative to the guinea pig maximization test. Controls are necessary for all tests.

6.3.1 These tests are for sensitization of the cell mediated type (Type IV). Since there are concerns about materials causing sensitization of the atopic type (Type I), measurement of IgE antibodies in test animals should also be considered. Similarly, measurement of IgE antibodies in humans in clinical trials may be considered.

6.4 *Skin Irritation Assay*—This is a patch test on the skin of rabbits, and after 24 hours the patches are removed and skin graded for erythema and edema. One available version is included in Practice F 719.

6.5 *Mucous Membrane Irritation*—The end use of the device product must be considered when deciding what tests to undertake. In some circumstances, the mucous membrane should be considered for the testing site. Numerous tests utilizing different mucous membranes and different animals have been reported. There remains some controversy about the applicability of the results of these tests to human clinical use. The material investigator should consider the appropriateness of a particular test site and published discussion of these methods when planning testing. The intracutaneous irritation test (see section 6.6) may be the more suitable test.

6.6 *Intracutaneous Injection (Irritation) Assay*—This assay is designed to determine biological response of rabbits to the single-dose intracutaneous injection of appropriate extracts prepared from test samples. All rabbits are observed for signs of erythema (tissue redness) and edema (tissue swelling) at the injection site for periods up to 72 h. Significant reactions are recorded and the test extract is graded. A USP test has been in use for many years, and Practice F 749 may be consulted for additional information.

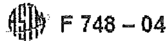
6.7 *Systemic Injection (Acute Toxicity) Assay*—This assay is designed to determine the biological response of animals (mice) to the single-dose intravenous or intraperitoneal injection of extracts prepared from test samples. The preferred extracts are saline, vegetable oil, or other liquids simulating body fluids or the vehicles of pharmaceutical products that may contact and potentially extract the material before reaching the patient. All mice are observed for signs of toxicity immediately after injection and again at specified intervals. Significant responses are recorded, and the test extract is graded. A USP procedure has been in use for many years, and many variations exist, including Practice F 750.

6.8 *Blood Compatibility*—Hemolysis and thrombosis are the most obvious examples of blood materials incompatibility, although adverse effects on plasma proteins, enzymes, and formed blood elements can also occur. One such method for screening for the adverse effects on formed blood elements is Practice F 2151. Thrombogenicity can be studied through specifically designed *in vitro*, or *ex vivo* procedures specific to the type of product being tested. Normally these tests are dynamic, simulated in-use procedures, with each being developed specifically by the organization interested in evaluating the device in question. Hemolysis is covered in 6.9.

6.9 *Hemolysis*—While hemolysis testing is frequently performed in combination with other tests for blood compatibility as specified in 6.8, several methods are in use whereby both materials and extracts are utilized for determining hemolysis. Test rods and extracts of the materials are incubated with human or rabbit blood in dynamic and static test tubes. The amount of plasma hemoglobin is measured and compared to reference materials and controls. Practice F 756 describes one method for the performance of hemolysis testing. In addition, hemolysis may be evaluated in finished devices by means of dynamic *in vitro*, *in vivo*, or *ex vivo* procedures designed to emphasize the hemolytic effect of the entire device. These tests tend to be proprietary to the various organizations who employ them.

6.10 *Complement Activation*—The interaction of blood with some materials, especially large surfaces (such as in dialysis membranes), may lead to the activation of the complement cascade leading to patient morbidity. Testing for activation of the various complement components using *in vitro* systems is available and recommended for blood contacting materials and devices. Two test methods may be found in Practices F 1984 and F 2065.

6.11 *Pyrogenicity*—Pyrogenic (fever producing) substances are either components of bacteria (gram negative predominately) or fungi (rarely) or are chemical in origin. The latter are most commonly known as "material-mediated" pyrogens. The most common causes of pyrogenicity are endotoxins or lipopolysaccharide (LPS) of gram negative bacterial cell wall membranes, which can be detected in the Limulus Amebocyte Lysate (LAL) test (USP bacterial endotoxin test). Endotoxins are also detected using the USP rabbit test, which will detect all types of pyrogens, including material-mediated pyrogens. Sterile devices that can be demonstrated as passing either the USP rabbit test or the LAL test are commonly labeled as "non-pyrogenic" and each batch of product is tested for pyrogenicity (unless a different schedule can be adopted based on historical data, process validation, or controls).



6.11.1 Since depyrogenating endotoxin-contaminated devices is difficult, costly, and often impractical, pyrogen testing is sometimes performed on incoming raw materials or components as a screening method. The LAL test should be used for LPS screening purposes before any rabbit test for material-mediated pyrogens. If the identities of possible material-mediated pyrogens are known, every effort should be made to detect material-mediated pyrogens by analytical or other means not involving the USP rabbit test.

6.12 *Implantation Tests*—The end-use application should be considered when choosing the most suitable site for testing.

6.12.1 *Short-Term Subcutaneous Implantation Test*—Since many implants are intended specifically for subcutaneous use, it is important to consider the reaction of this tissue space to implants and materials. The potential for mobility of implants and tissue of the subcutaneous plane makes this site significantly different from other tissue implantation sites. Inflammatory responses may be increased with motion. Practice F 1408 provides one method for short-term implant testing in a subcutaneous site.

6.12.2 *Short-Term Intramuscular Implantation Test*—This type of test is designed to evaluate the reaction of living tissue to a sample material that is surgically implanted into animal tissue (preferably the rabbit, but larger animals (such as the dog) may be considered where necessary). At the conclusion of the assay period, the sites of implantation are examined for significant reaction, and the test material is graded. A USP test has been in use for many years and 7- and 30-day evaluation is available in Practice F 763.

6.12.3 *Implantation Testing for the Biological Response to Particles*—Practice F 1904 is an intermediate-term test to evaluate the unique responses that may occur when materials are introduced in a particulate form or are reduced to particulate form as a result of the mechanical actions of device utilization.

6.12.4 *Long-Term Implant Test*—Practice F 981 is a long-term implantation test in muscle and bone for metals, plastics, and ceramics. In the case of absorbable/resorbable implant materials, Practice F 1983 should be considered as an alternative to or in addition to Practice F 981. Other long-term implant tests may be appropriate for long-term implant applications.

6.13 *Genotoxicity*—A number of tests are available to assess genotoxic potential. The Ames test may be used as a preliminary screening study with materials. Methods that have been developed for genotoxicity testing in mammalian cells are included in Guides E 1202, E 1262, E 1263, and E 1280 and Practices E 1397 and E 1398. Additionally, other tests may be suggested by regulatory agencies for certain implant applications and sites. No single test yet developed can detect all types of mutagens.

6.14 *Carcinogenicity*—Carcinogenicity testing is usually quite specific for the test substance, with no standard procedures available at this time. Guide F 1439 provides guidelines for the performance of these types of tests on implant materials. The National Toxicology Program has published a very comprehensive document[5] relating to the conduct of carcinogenicity testing of chemicals. While much of this document may not be applicable to implant materials, many of the recommendations for animal care, selection of model, and methods for ensuring the integrity of data may be applicable. The user of this document should be aware that very little is known about the latency periods for the development of tumors due to implant materials in the human or the relationship between the results of animal testing and the long-term clinical response. The primary measure of the carcinogenic potential of implant materials will be the results of long term clinical use.

6.15 *Immunotoxicity*—Materials may influence the immune system of the host in various ways. There may be toxicity to the cells in the immune system resulting in decreased responsiveness to antigens. There may be stimulation of the immune system resulting in increased immune responses to antigens. There may be stimulation of an immune response to components or extracts of the materials, which may or may not result in patient morbidity or unsatisfactory performance of the device. Testing for immunotoxicity and specific immune responses may be considered, especially for materials of natural origin or materials that are oil, wax, or gel in nature. Two such methods that may be considered are Practices F 1905 and F 1906.

6.16 *Batch Testing of Materials and Devices for Biocompatibility*—Biocompatibility testing of materials may, in some circumstances, be done on samples from a batch of material to be used and the methods used for testing depend on the type of industry, product, and manufacturing and quality control operations in use. Periodic biocompatibility audits may be performed, depending on the manufacturer's degree of assurance that the supplier will not change his product or process, intentionally or otherwise. Additional biocompatibility testing must be performed when changes are made in the composition or processing of the materials.

7. Keywords

7.1 animal testing; biocompatibility; *in vivo* testing; laboratory testing; toxicity

[5] General Statement of Work for the Conduct of Toxicology and Carcinogenicity Studies in Laboratory Animals, *National Toxicology Program*, April 1987.

3．欧州における規格

（1）EC 指令の概要

EC において、製品を流通させる場合には EC 指令に従い、CE マークを取得する必要がある。EC 指令は、人々の基本的な健康や安全に関する要件を定め、製品の適合性の評価方法を確立するためにつくられた EU 内の法的義務を生じさせる規律であり、域内の技術的整合・規格に関し、統合の障壁を排除することを目的としている。策定機関は EU 理事会であり、対象は、EU 域内で流通させる製品（製造業者・販売業者）である。この EC 指令に従い、EU 域内（EU/EEA）で商取引を行う製品へ CE マーク（適合性表示マーク）を表示する義務が生じる。EC 指令の例として、玩具指令、低電圧指令、機械指令、EMC 指令、防爆機器指令などがあり、その一つとして、医療機器に関する指令（Medical Devices Directive : Council Directive 93/42/EEC）が存在する。再生医療に関する最終製品についても、EU 域内で流通させようとする場合には、この指令に従い CE マークを取得する必要性があると言える。医療機器指令の条文構成は以下の表のとおりである。

表 7 医療機器指令条文構成

条文（Article）	付属書（Annex）
３条：製品の基本要件への適合	付属書１：基本要件（製品として必ず満たさなければならない要求事項）
５条：基本要件への適合を示す整合規格	
８条：保護措置	―
９条：製品のリスクによるクラス分類	付属書９：クラス分類基準
１０条：市場投入後に起こる事故に関する情報	
１１条：製品のリスクの度合いに応じた適合性評価手順及び適合宣言	付属書２－７：適合性評価手順 付属書８：カスタムメード機器、臨床評価用機器
１４条：市場出荷責任者の登録、製造業者の登録事務所及びＥＣ域内責任者	―
１５条：臨床試験	付属書１０：臨床評価
１６条：通知機関	付属書１１：通知機関認定基準
１７条：ＣＥマーキング	付属書１２：ＣＥマーキング

（2）EN 規格

1）概要

医療機器指令では、安全性確保の観点から必須要求事項が定められており、CE マーク取得にはその要件を満たす必要がある。

表 8 医療機器指令の必須要求事項

Ⅰ．全般的要件	１．患者の健康・安全を考慮した設計・製造
	２．設計の方法は一般に認められた技術水準を考慮し、安全原則に適合
	３．意図する性能を達成するための設計、製造及び包装
	４．患者又は使用者の健康や安全を脅かす程に悪影響を与えない設計・製造
	５．輸送及び保管条件下における性能劣化を防止する設計・製造
	６．有効性が起こりうる不具合を上回る設計・製造
Ⅱ．設計及び組立てに関する要件	７．化学的・物理的及び生物学的特性（例：生体適合性）
	８．感染及び細菌汚染（例：包装、滅菌）
	９．構造及び環境特性（例：他の機器との組み合わせ使用時の安全性）
	１０．測定機能を有する機器（例：規定法定単位の使用）
	１１．放射線に関する防護
	１２．エネルギー源に接続される機器またはエネルギー源を有する機器に関する要件
	１３．製造業者が供給する情報（表示・ラベル・取扱説明書 等）
	１４．臨床データによる実証

もっとも、指令における要件は、製品の技術的な仕様を定めたものではなく、一般的な内容を示すのみであるため、実際には、欧州標準化委員会（CEN）が策定した欧州規格（EN規格）、またはこの規格に準拠して採択されたEU各加盟国の国内規格において定められた具体的な技術的条件を満たす必要がある。EN規格は任意の規格であり、採用するかどうかは製造業者や流通業者に任されているが、このEN規格の条件を満たすことがEC指令の必須要求事項を満たしていることとみなされるため、実質的にはEN規格に拘束力が生じている。

２）運営体制

CENは欧州における最大の任意標準化機関である。CENはEEC（欧州経済共同体：European Economic Community）及びEFTA（欧州自由貿易連合会：European Free Trade Association）諸国の国家規格機関の連合として設立され、2003年現在で28カ国のメンバーで構成されている。CENは、欧州規格と関連試験方法の作成を担当する専門委員会の事務局を提供している。CENにおける主要な組織を下図に示す。

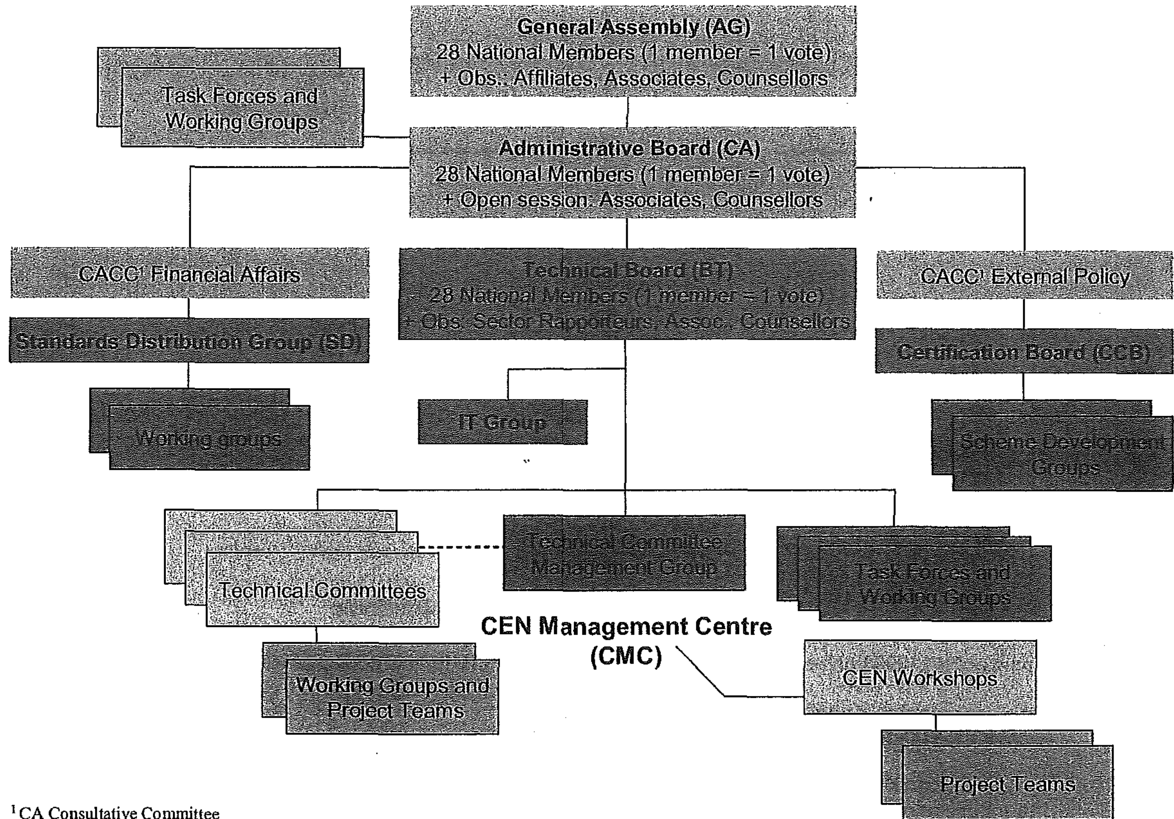


図 3 CEN における主要な組織[7]

（3） 規格の具体的内容

上述した EN 規格においては、CEN が独自に作成した規格や、CENELE（欧州電気標準化委員会）による規格、AECMA（European Association of Aerospace Industries）による規格、などにより構成されているが、EN 規格においては、国際機関である ISO 規格を踏襲し EN 規格として策定している規格も存在する。特に、再生医療分野に大きく関係する生物学的安全性の評価に関する規格については、ISO に基づく規格として採用されており、EN30993 の規格が存在する。この EN30993 では、各国での採用状況によりタイトル等は若干異なるものの、その具体的な内容は ISO とほぼ同様である。その具体的内容の例を以下に挙げる。

[7] CEN(
http://www.cenorm.be/cenorm/aboutus/structure+/thecensystem/thecensystem.asp)参照。

(a) EN30993-1

EN30993-1 では、医療機器に対して必要な生物学的試験が説明されている。これは、ISO10993-1 と同等の内容を持つ規格である。以下、その内容を示す。

EN 30 993-1:1994

Biological evaluation of medical devices —

Part 1:
Guidance on selection of tests

1 Scope

This part of ISO 10993 gives guidance on

a) the fundamental principles governing the biological evaluation of medical devices;

b) the definition of categories of devices based on the nature and duration of contact with the body;

c) the selection of appropriate tests.

ISO 10993 does not cover testing of materials and devices that do not come into contact with the patient's body directly or indirectly. Nor does it cover biological hazards arising from any mechanical failure. Other parts of ISO 10993 cover specific tests as indicated in the foreword. (See A.2, clause 1 Scope.)

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this part of ISO 10993. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this part of ISO 10993 are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 9001:1987, *Quality systems — Model for quality assurance in design/development, production, installation and servicing.*

ISO 9004:1987, *Quality management and quality system elements — Guidelines.*

3 Definitions

For the purposes of ISO 10993, the following definitions apply.

3.1 medical device: Any instrument, apparatus, appliance, material or other article, including software, whether used alone or in combination, intended by the manufacturer to be used for human beings solely or principally for the purpose of:

- diagnosis, prevention, monitoring, treatment or alleviation of disease, injury or handicap;
- investigation, replacement or modification of the anatomy or of a physiological process;
- control of conception;

and which does not achieve its principal intended action in or on the human body by pharmacological, immunological or metabolic means, but which may be assisted in its function by such means.

NOTES

1 Devices are different from drugs and their biological evaluation requires a different approach.

2 Use of the term "medical device" includes dental devices.

3.2 material: Any synthetic or natural polymer, metal, alloy, ceramic, or other nonviable substance, including tissue rendered nonviable, used as a device or any part thereof.

3.3 final product: Medical device in its "as used" state.

4 General principles applying to biological evaluation of materials and devices

4.1 *In the selection of materials to be used in device manufacture, the first consideration should be fitness for purpose having regard to the characteristics and properties of the material, which include chemical, toxicological, physical, electrical, morphological and mechanical properties.*

4.2 The following should be considered for their relevance to the overall biological evaluation of the device:

a) the material(s) of manufacture;

b) intended additives, process contaminants and residues;

c) leachable substances;

d) degradation products;

e) other components and their interactions in the final product;

f) the properties and characteristics of the final product.

NOTE 3 If appropriate, identification and quantification of extractable chemical entities of the final product should precede biological evaluation.

4.3 Tests and their interpretation to be used in the biological evaluation should take into account the chemical composition of the materials including the conditions of exposure as well as the nature, degree, frequency and duration of the device or its constituents to the body. By following these principles devices can be categorized to facilitate the selection of appropriate tests. This guide is concerned with the tests to be carried out on materials and/or the final product.

The range of potential hazards is wide and may include:

a) short-term effects (e.g., acute toxicity, irritation to the skin, eye and mucosal surfaces, sensitization, haemolysis and thrombogenicity);

b) long-term or specific toxic effects (e.g., subchronic and chronic toxic effects, sensitization, genotoxicity, carcinogenicity (tumorigenicity) and effects on reproduction including teratogenicity).

4.4 All potential biological hazards should be considered for every material and final product but this does not imply that testing for all potential hazards will be necessary or practical (see clause 7).

4.5 Any *in vitro* or *in vivo* tests shall be based on end-use applications and appropriate good laboratory practice followed by evaluation by competent informed persons. Whenever possible, *in vitro* screening should be carried out before *in vivo* tests are commenced. Test data, complete to the extent that an independent analysis conclusion could be made, should be retained.

4.6 The materials or final product shall be considered (see A.2, subclause 4.6) for biological reevaluation if any of the following occurs:

a) any change in the source or in the specification of the materials used in the manufacture of the product;

b) any change in the formulation, processing, primary packaging or sterilization of the product;

c) any change in the final product during storage;

d) any change in the intended use of the product;

e) any evidence that the product may produce adverse effects when used in humans.

4.7 The biological evaluation performed in accordance with this part of ISO 10993 should be considered in conjunction with the nature and mobility of the ingredients in the materials used to manufacture the device and other information, other nonclinical tests, clinical studies, and post-market experiences for an overall assessment (see A.2, subclause 4.7).

5 Categorization of medical devices

The testing of any device that does not fall into one of the following categories should follow the general principles contained in this part of ISO 10993. Certain devices may fall into more than one category, in which case testing appropriate to each category should be considered.

5.1 Categorization by nature of contact

5.1.1 Non-contact devices

These are devices that do not contact the patient's body directly or indirectly and are not included in ISO 10993.

5.1.2 Surface-contacting devices

These include devices in contact with the following:

a) **skin:** devices that contact intact skin surfaces only; examples include electrodes, external

prostheses, fixation tapes, compression bandages and monitors of various types;

b) **mucosal membranes:** devices communicating with intact mucosal membranes; examples include contact lenses, urinary catheters, intravaginal and intraintestinal devices (stomach tubes, sigmoidoscopes, colonoscopes, gastroscopes), endotracheal tubes, bronchoscopes, dental prostheses, orthodontic devices and IUDs;

c) **breached or compromised surfaces:** devices that contact breached or otherwise compromised body surfaces; examples include ulcer, burn, and granulation tissue dressings or healing devices and occlusive patches.

5.1.3 External communicating devices

These include devices communicating with the following:

a) **blood path, indirect:** devices that contact the blood path at one point and serve as a conduit for entry into the vascular system; examples include solution administration sets, extension sets, transfer sets and blood administration sets;

b) **tissue/bone/dentin communicating:** devices and materials communicating with tissue, bone and pulp/dentin system; examples include laparoscopes, arthroscopes, draining systems, dental cements, dental filling materials and skin staples;

c) **circulating blood:** devices that contact circulating blood; examples include intravascular catheters, temporary pacemaker electrodes, oxygenators, extracorporeal oxygenator tubing and accessories, dialyzers, dialysis tubing and accessories, haemoadsorbents and immunoadsorbents.

5.1.4 Implant devices

These include devices in contact with the following:

a) **tissue/bone:** devices principally contacting bone; examples include orthopaedic pins, plates, replacement joints, bone prostheses, cements and intraosseous devices. Devices principally contacting tissue and tissue fluid; examples include pacemakers, drug supply devices, neuromuscular sensors and stimulators, replacement tendons, breast implants, artificial larynxes, subperiosteal implants and ligation clips;

b) **blood:** devices principally contacting blood; examples include pacemaker electrodes, artificial arteriovenous fistulae, heart valves, vascular grafts, internal drug delivery catheters and ventricular assist devices.

5.2 Categorization by duration of contact

Contact duration may be categorized as follows:

a) **limited exposure (A):** devices whose single or multiple use or contact is likely to be up to 24 h;

b) **prolonged exposure (B):** devices whose single, multiple or long-term use or contact is likely to exceed 24 h bot not 30 days;

c) **permanent contact (C):** devices whose single, multiple or long-term use or contact exceeds 30 days.

If a material or device may be placed in more than one duration category, the more rigorous testing requirements should apply. With multiple exposures, the decision into which category a device is placed should take into account the potential cumulative effect, bearing in mind the period of time over which these exposures occur.

6 Testing

6.1 General

In addition to the general principles laid down in clause 4, the following should be applied to biological testing of medical devices.

a) Testing should be performed on the final product, or representative samples from the final product or materials.

b) The choice of test procedures shall take into account:

   1) the nature, degree, duration, frequency and conditions of exposure to or contact of humans to the device in the normal intended use;

   2) the chemical and physical nature of the final product;

   3) the toxicological activity of the chemicals in the formulation of the final product;

   4) that certain tests (e.g., those designed to assess systemic effects) may not be applicable where the presence of leachable materials has been excluded, or where leachables have a known and acceptable toxicity profile;

   5) the relationship of device surface area to recipient body size;

   6) the existing information based on the literature, experience and non-clinical tests;

7) the protection of humans is the primary goal of this document; a secondary goal is to ensure animal welfare and to minimize the number and exposure of animals.

c) If extracts of the devices are prepared, the solvents and conditions of extraction used should be appropriate to the nature and use of the final product.

d) Positive and negative controls should be used where appropriate.

e) Test results cannot ensure freedom from potential biological hazard, thus biological investigations should be followed by careful observations for unexpected adverse reactions or events in humans during clinical use of the device.

Annex B provides a Bibliography of International Standards and Guidelines on biological response test methods.

6.2 Initial evaluation tests

The initial biological response tests that should be considered are as given in 6.2.1 to 6.2.9.

6.2.1 Cytotoxicity

With the use of cell culture techniques, these tests determine the lysis of cells (cell death), the inhibition of cell growth, and other effects on cells caused by devices, materials and/or their extracts.

6.2.2 Sensitization

These tests estimate the potential for contact sensitization of devices, materials and/or their extracts, using an appropriate model. These tests are appropriate because exposure or contact to even minute amounts of potential leachables can result in allergic or sensitization reactions.

6.2.3 Irritation

These tests estimate the irritation potential of devices, materials and/or their extracts, using appropriate site or implant tissue such as skin, eye and mucous membrane in a suitable model. The test(s) performed should be appropriate for the route (skin, eye, mucosa) and duration of exposure or contact to determine irritant effects of devices, materials and potential leachables.

6.2.4 Intracutaneous reactivity

These tests assess the localized reaction of tissue to device extracts. These tests are applicable where determination of irritation by dermal or mucosal tests are inappropriate (e.g., devices having access to the blood path). These tests may also be useful where extractables are hydrophobic.

6.2.5 Systemic toxicity (acute)

These tests estimate the potential harmful effects of either single or multiple exposures, during a period of less than 24 h, to devices, materials and/or their extracts in an animal model. These tests are appropriate where contact allows potential absorption of toxic leachables and degradation products.

Pyrogenicity tests are included to detect material-mediated pyrogenic reactions of extracts of devices or materials. No single test can differentiate pyrogenic reactions that are material-mediated from those due to endotoxin contamination.

6.2.6 Sub-chronic toxicity (sub-acute toxicity)

These tests determine the effects of either single or multiple exposures or contact to devices, materials and/or their extracts during a period of not less than 24 h to a period not greater than 10 % of the total life-span of the test animal (e.g., up to 90 days in rats). These tests may be waived for materials with chronic toxicity data. The reason for waiving of the tests should be included in the final report. These tests should be appropriate for the route and duration of contact.

6.2.7 Genotoxicity

These tests apply mammalian or non-mammalian cell culture or other techniques to determine gene mutations, changes in chromosome structure and number, and other DNA or gene toxicities caused by devices, materials and/or their extracts.

6.2.8 Implantation

These tests assess the local pathological effects on living tissue, at both the gross level and microscopic level, of a sample of a material or final product that is surgically implanted or placed into an implant site or tissue appropriate to the intended application (e.g., special dental usage tests have been described). These tests should be appropriate for the route and duration of contact. For a material, these tests are equivalent to sub-chronic toxicity tests if systemic effects are also investigated.

6.2.9 Haemocompatibility

These tests evaluate effects on blood or blood components by blood-contacting devices, materials or using an appropriate model or system. Specific haemocompatibility tests may also be designed to simulate the geometry, contact conditions and flow dynamics of the device or material during clinical applications.

Haemolysis tests determine the degree of red blood cell lysis and the release of haemoglobin caused by devices, materials and/or their extracts *in vitro*.

6.3 Supplementary evaluation tests

The supplementary biological evaluation tests that should be considered are as given in 6.3.1 to 6.3.4.

6.3.1 Chronic toxicity

These tests determine the effects of either single or multiple exposures to devices, materials and/or their extracts during a period of at least 10 % of the life-span of the test animal (e.g., over 90 days in rats). These tests should be appropriate for the route and duration of exposure or contact.

6.3.2 Carcinogenicity

These tests determine the tumorigenic potential of devices, materials and/or their extracts from either a single or multiple exposures or contacts over a period of the total life-span of the test animal. These tests may be designed in order to examine both chronic toxicity and tumorigenicity in a single experimental study. Carcinogenicity tests should be conducted only if there are suggestive data from other sources. These tests should be appropriate for the route and duration of exposure or contact.

6.3.3 Reproductive and developmental toxicity

These tests evaluate the potential effects of devices, materials and/or their extracts on reproductive function, embryonic development (teratogenicity), and prenatal and early postnatal development. Reproductive/developmental toxicity tests or bioassays should only be conducted when the device has potential impact on the reproductive potential of the subject. The application site of the device should be considered.

6.3.4 Biodegradation

Where the potential for resorption and/or degradation exists, such tests may determine the processes of absorption, distribution, biotransformation, and elimination of leachables and degradation products of devices, materials and/or their extracts.

7 Guidance on selection of biological evaluation tests

Table 1 identifies the initial evaluation tests that shall be considered for each device and duration category. Table 2 identifies the supplementary evaluation tests that shall be considered for each device and duration category.

Due to the diversity of medical devices, it is recognized that not all tests identified in a category will be necessary or practical for any given device. It is indispensable for testing that each device shall be considered on its own merits: additional tests not indicated in the table may be necessary.

It is strongly recommended that the rationale for selection and/or waiving of tests be recorded.

8 Assurance of test methods

8.1 Test method assurance

The test methods used in the biological evaluation shall be sensitive, precise, and accurate. The test results should be reproducible (interlaboratory) as well as repeatable (intralaboratory).

8.2 Continued assurance

The assurance that a material is initially acceptable for its intended use in a product, and its continued acceptability in the long term, is an aspect of a quality system. (See A.2, subclause 8.2.)

ISO 9001:1987, clause 4 specifies the requirements for such quality assurance systems. ISO 9004 provides more detailed guidance for designing and manufacturing products.

Table 1 — Guidance for initial evaluation tests

NOTE — Each device shall be considered on its own merits.

Device categories			Biological effect							
Body contact (see 5.1)		Contact duration (see 5.2) A-limited (≤ 24 h) B-prolonged (> 24 h to 30 days) C-permanent (> 30 days)	Cytotoxicity	Sensitization	Irritation or intracutaneous reactivity	Systemic toxicity (acute)	Sub-chronic toxicity (sub-acute toxicity)	Genotoxicity	Implantation	Haemocompatibility
Surface devices	Skin	A	X	X	X					
		B	X	X	X					
		C	X	X	X					
	Mucosal membranes	A	X	X	X					
		B	X	X	X					
		C	X	X	X		X	X		
	Breached or compromised surfaces	A	X	X	X					
		B	X	X	X					
		C	X	X	X		X	X		
External communicating devices	Blood path, indirect	A	X	X	X	X				X
		B	X	X	X	X				X
		C	X	X		X	X	X		X
	Tissue/bone/dentin communicating	A	X	X	X					
		B	X	X				X	X	
		C	X	X				X	X	
	Circulating blood	A	X	X	X	X				X
		B	X	X	X	X		X		X
		C	X	X	X	X	X	X		X
Implant devices	Tissue/bone	A	X	X	X					
		B	X	X				X	X	
		C	X	X				X	X	
	Blood	A	X	X	X	X			X	X
		B	X	X	X	X		X	X	X
		C	X	X	X	X	X	X	X	X

Table 2 — Guidance for supplementary evaluation tests

NOTE — Each device shall be considered on its own merits.

Device categories		Biological tests				
Body contact (see 5.1)		Contact duration (see 5.2) A-limited (≤ 24 h) B-prolonged (> 24 h to 30 days) C-permanent (> 30 days)	Chronic toxicity	Carcinogenicity	Reproductive/developmental	Biodegradation
Surface devices	Skin	A				
		B				
		C				
	Mucosal membranes	A				
		B				
		C				
	Breached or compromised surfaces	A				
		B				
		C				
External communicating devices	Blood path, indirect	A				
		B				
		C	X	X		
	Tissue/bone/dentin communicating	A				
		B				
		C		X		
	Circulating blood	A				
		B				
		C	X	X		
Implant devices	Tissue/bone	A				
		B				
		C	X	X		
	Blood	A				
		B				
		C	X	X		

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SNP Communications

Genetic Variations and Haplotypes of CYP2C19 in a Japanese Population

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: Forty-eight single nucleotide variations, including 27 novel ones, were found in the 5′-regulatory region, all of the exons and their surrounding introns of *CYP2C19* in 253 Japanese subjects (134 diabetic patients and 119 healthy volunteers). Identified novel variations were as follows: −2772G>A, 2767_−2760delGGTGAACA, −2720T>C, −2547delG, −2545G>T, −2545_−2544 delGC, and −2040C>T in the enhancer region; −778C>T, −777G>A, −529G>C, −189C>A, and −185A>G in the promoter region; 151A>G (S51G), 481G>C (A161P), 986G>A (R329H), 1078G>A (D360N), and 1119C>T (D373D) in the exons, and IVS1+128T>A, IVS3+163G>A, IVS4+271A>G, IVS5−49A>G, IVS6−210C>T, IVS6−196T>A, IVS6−32T>A, IVS7+84G>A, IVS7 −174C>T, and IVS8+64C>T in the introns. Since we found no significant differences in the variation frequencies between healthy volunteers and diabetic patients, the data for all subjects were treated as one group in further analysis. The allele frequencies were 0.265 for IVS6−196T>A, 0.045 for −2772G>A and −2720T>C, 0.024 for −2040C>T, 0.014 for IVS7−174C>T, 0.010 for −529G>C, 0.006 for IVS1+128T>A and 481G>C (A161P), 0.004 for −2767_−2760delGGTGAACA and IVS6−210C>T, and 0.002 for the other 17 variations. In addition, the two known nonsynonymous single nucleotide polymorphisms, 681G>A (splicing defect, *2 allele) and 636G>A (W212X; **3* allele) were detected at 0.267 and 0.128 frequencies, respectively. No variation was detected in the known binding sites for constitutive androstane receptor and glucocorticoid receptor. Linkage disequilibrium analysis showed several close linkages of variations throughout the gene. By using the variations, thirty-one haplotypes of *CYP2C19* and their frequencies were estimated. Our results would provide fundamental and useful information for genotyping *CYP2C19* in the Japanese and probably other Asian populations.

Key words: CYP2C19; genetic variation; amino acid alteration; haplotype

On June 13, 2005, these variations were not found on the homepage of the CYP Allele Nomenclature Committee (http://www.imm.ki.se/CYPallele/CYP2C19), the Japanese Single Nucleotide Polymorphisms (JSNP) (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB (http://www.pharmgkb.org/do/) database.

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Introduction

In human, cytochrome P450 2C (CYP2C) subfamily consists of four members: CYP2C8, CYP2C9, CYP2C18 and CYP2C19. Their genes are tandemly located on chromosome 10q24.[1] One of the subfamily members, CYP2C19 is a clinically important enzyme that metabolizes a wide variety of drugs, such as antiulcer drugs omeprazole and lansoprazole, an anticonvulsant *S*-mephenytoin, an antidiabetic drug tolbutamide, and an anxiolytic drug diazepam.[2,3] Metabolism of these drugs *in vivo* has been known to be polymorphic, and individuals can be divided into extensive metabolizers and poor metabolizers (PMs). PMs would face higher area under concentration-time curve (AUC) values of the drugs.[4-6] Regarding omeprazole, for example, the PMs show higher cure rates for *Helicobacter pyloli* infection and peptic ulcer because of the higher AUCs, which lead to increased gastric pH and thus are suggested to result in stable antibacterial activity of amoxicillin.[2,4,7]

Ethnic differences in the incidence of PMs among Caucasians (2–5%), Africans (*ca.* 6%) and Asians (13–23%) have been reported.[2,8] These differences are known to be attributed to the genetic polymorphisms of *CYP2C19* gene. de Morais *et al.* first reported the common single nucleotide polymorphisms (SNPs) 681G>A (splicing defect, *CYP2C19*2* allele) and 636G>A (W212X, **3* allele), the latter of which was found only in Japanese but not in Caucasian populations.[9,10] Recent studies showed that the **3* allele is also distributed in Chinese, Thai and Vietnamese with different frequencies.[11] *CYP2C19*2* and **3* generate the null-activity enzyme protein and have been considered to account for >99% of PM alleles in the Japanese population.[10,12] Recently, another minor allele, *CYP2C19*16* (1324C>T, R442C), was found in a Japanese subject who had received mephobarbital (at 0.006 frequency in Japanese population).[13] As for other Asian populations, the two defective alleles *CYP2C19*4* (1A>G, no protein) and **5* (1297C>T, R433W) were also found below 0.005 frequencies in Chinese.[14,15] However, the other alleles (*CYP2C19*6*-**15*) have not been detected in Asians.

Recently, the transcriptional regulatory regions of the *CYP2C19* gene were analyzed. Reporter assay with up to 1.8 kb upstream of the 5′-flanking region showed that there were potentially negative and positive elements between 650 to 453 bases and between 224 to 17 bases, respectively, upstream of the translational start site.[16] Furthermore, another report revealed enhancer elements for constitutive androstane receptor (−1891 to −1876 bases from the translational start site) and glucocorticoid receptor (−1750 to −1736).[17]

While the effects of *CYP2C19* polymorphisms have been extensively studied on the **2* and **3* polymorphisms, a comprehensive search for genetic polymorphisms of *CYP2C19* in Asian populations, including the Japanese, is currently lacking. In this study, the 5′-regulatory region, all the exons and their surrounding introns of *CYP2C19* were sequenced in 253 Japanese subjects, and 27 novel variations, including four nonsynonymous ones, were identified.

Materials and Methods

Human genomic DNA samples: DNA was extracted from the blood leukocytes of 134 Japanese diabetic patients who had received glimepiride. DNA was also extracted from Epstein-Barr virus-transformed lymphoblastoid cells, which were derived from blood samples collected from 119 healthy Japanese volunteers at the Tokyo Women's Medical University. The ethical review boards of the International Medical Center of Japan, the Nerima General Hospital, the Tokyo Women's Medical University, and the National Institute of Health Sciences approved this study. Written informed consent was obtained from all participating patients as well as all healthy subjects.

Polymerase chain reaction (PCR) conditions for DNA sequencing: First, the multiplex PCR was performed to amplify the entire *CYP2C19* gene by the two mixed primer sets (Mix 1 and Mix 2 in "1st PCR" in **Table 1**). Amplification was performed from 100 ng of genomic DNA using 1.25 units of Ex-Taq (Takara Bio. Inc, Shiga, Japan) with the 0.2 μM of the primers sets. The first PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. Then each exon was amplified separately using one-fifth volume of the 1st PCR product as a template by Ex-Taq (0.625 units) (Takara Shuzo) with a set of primers (0.2 μM) listed in "2nd PCR" of **Table 1** (designed in the intronic regions). The second-round PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 7 min. Thereafter, the PCR products were treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the primers listed in "Sequencing" of **Table 1**. The excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany). The eluates were analyzed on an ABI Prism 3730 DNA Analyzer (Applied Biosystems). All the detected variations were confirmed by repeating the PCR from the genomic DNA and sequencing the newly generated PCR products.

Linkage disequilibrium (LD) and haplotype analysis: Hardy-Weinberg equilibrium and LD analysis was

Table 1. Primer sequences used for the analysis of the *CYP2C19* gene

		Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified length (bp)
1st PCR	Mix 1	−3k to Exon 1	TTATTTGTTGCTAGGGCTCGTG	CTTACTGTTTACCCTCAGCC	3,281
		Exon 2 to 3	AGGTAGACACAAGAGTGCTGA	TTCTCTGGTGACATGTTCTGGA	1,250
	Mix 2	Exon 4 to 5	CCATTATTTAACCAGCTAGGC	TCCTATCCTGACATCCTTATTG	1,969
		Exon 6	CAAACTATACCAAACAGCAGGCTA	ACTCTACATAGCTTAAAGGGCTCA	6,557
		Exon 7	AATGCTGAAGTGGGTTGTTG	ACCCTGACAGAAATTCTAGCCC	1,272
		Exon 8 to 9	CCCACAACAGTCCCCGAA	CACAAAGGAAGGAAGGTCTAA	3,650
2nd PCR		−3K	TTATTTGTTGCTAGGGCTCGTG	ATCACATCCCGTCTCATAGAA	476
		−2K	CTCAACTTAGCAGAAGAGAGG	CTCATATCCCTTTGGAATCTCT	562
		−1K	AAGCCTTAGTTTCTCAAGCCC	CTTGTTCTCCTTCGTCCCAG	925
		Exon 1	AGAAGACCTCAGCTCAAATCC	CTTACTGTTTACCCTCAGCC	1,249
		Exon 2 to 3	AGGTAGACACAAGAGTGCTGA	TTCTCTGGTGACATGTTCTGGA	1,250
		Exon 4	CCATTATTTAACCAGCTAGGC	AGCCTTGTGAGTAATGGAAGA	727
		Exon 5	AGAAGTCATTTAACTGCTCTGG	TCCTATCCTGACATCCTTATTG	950
		Exon 6	CTCTCTCACCGCTCCTATTCA	GCTGGGATTACAGTGGTGTG	627
		Exon 7	GGTCTTGTTTCTTCATCTAGTCAG	ACCCTGACAGAAATTCTAGCCC	915
		Exon 8	CCCACAACAGTCCCCGAA	GAGGATGTATCACCAGCGGAG	580
		Exon 9	TTGTTTAGTTGCCTATCCATCC	CACAAAGGAAGGAAGGTCTAA	775
Sequencing		−3K	TTATTTGTTGCTAGGGCTCGTG	ATCACATCCCGTCTCATAGAA	
		−2K	CTCAACTTAGCAGAAGAGAGG	CTCATATCCCTTTGGAATCTCT	
		−1K	TGTCTTCTGTTCTCAAAGCATC	CTGAATATATACCACATTCATCC	
		Exon 1	AGGCTGCTGTATTTTTAGTAGG	GACACTGACAGACTGGAAAAGG	
		Exon 2	AGGTAGACACAAGAGTGCTGA	GAGAAACGAAACTAGGAGG	
		Exon 3	GTTTCAGCATCTGTCTTGG	TTCTCTGGTGACATGTTCTGGA	
		Exon 4	CCATTATTTAACCAGCTAGGC	AGCCTTGTGAGTAATGGAAGA	
		Exon 5	AGAGGCTGCTTGATAGAAAT	TCCTATCCTGACATCCTTATTG	
		Exon 6	CTCACCGCTCCTATTCAATATT	AGTGGTGTGCCACAATGC	
		Exon 7	GGTCTTGTTTCTTCATCTAGTCAG	ACCCTGACAGAAATTCTAGCCC	
		Exon 8	CCCACAACAGTCCCCGAA	GAGGATGTATCACCAGCGGAG	
		Exon 9	TTGTTTAGTTGCCTATCCATCC	CACAAAGGAAGGAAGGTCTAA	

performed by SNPAlyze software (Dynacom Co., Yokohama, Japan), and pairwise LD between variations was analyzed by rho square (r^2) values. Some of the haplotypes were unambiguous from subjects with homozygous variations at all sites or a heterozygous variation at only one site. Separately, the diplotype configurations (a combination of haplotypes) were inferred by LDSUPPORT software, which determines the posterior probability distribution of the diplotype configuration for each subject based on the estimated haplotype frequencies.[18] The diplotype configurations of all subjects had a probability (certainty) of more than 0.975 except for 5 subjects. The haplotypes inferred in single subjects are described with haplotype names and a question mark in **Table 3**, since the predictability for these very rare haplotypes is known to be low in some cases. The haplotypes detected in this study were tentatively named as the numbers plus small alphabetical letters, except for the four haplotypes with novel nonsynonymous variations (*CYP2C19*18-*21*), which were registered to the CYP Allele Nomenclature Committe and will be publicized on the committee's Web site.

Results and Discussion

The enhancer (from 2780 to 2350 bases and from 2090 to 1590 bases upstream of the translational start site) and promoter regions (up to 1020 bases upstream of the translational start site), all the 9 exons and their flanking introns of *CYP2C19* were sequenced in 253 Japanese subjects. Genbank accession number NT_030059.12 was utilized for the reference sequence. Forty-eight genetic variations, including 27 novel ones (7 were in the enhancer region, 5 in the promoter region, 5 in the exons and 10 in the introns), were detected **(Table 2)**. Since we did not find any significant differences in the frequencies of these variations between healthy volunteers and diabetic patients (by χ^2 test or Fisher's exact test, $P>0.25$), the data for all subjects were analyzed as one group. All of the detected variations were found in Hardy-Weinberg equilibrium ($P\geqq 0.449$).

Four novel nonsynonymous variations, 151A>G (S51G), 481G>C (A161P), 986G>A (R329H) and 1078G>A (D360N), were found as individual heterozygotes at 0.002, 0.006, 0.002 and 0.002 frequencies, respectively **(Fig. 1)**. Among them, A161 and D360 are

Table 2. Summary of *CYP2C19* variations detected in a Japanese population

SNP ID				Position					Number of subjects			Frequency		
This Study	JSNP	dbSNP (NCBI)	Reference	Location	NT_030059.12	From the translational initiation site or from the end of nearest exon	Nucleotide change and flanking sequences (5′ to 3′)	Amino acid change	Wild-type	Hetero-zygote	Homo-zygote	Total (n=253)	Healthy volunteers (n=119)	Diabetic patients (n=134)
MPJ6_2C19_001[a]				Enhancer	15268217	−2772	GCTCGTGAGGAAG/AAGAGGGTGAACA		230	23	0	0.045	0.055	0.037
MPJ6_2C19_002[a]				Enhancer	15268222_15268229	−2767_−2760	TGAGGAAGAGAGGGTGAACA/-GGTAGAGCACAG		251	2	0	0.004	0.000	0.007
MPJ6_2C19_003[a]				Enhancer	15268269	−2720	GTAAAGCTATTTT/CATATGATACTAC		230	23	0	0.045	0.055	0.037
MPJ6_2C19_004[a]				Enhancer	15268442	−2547	GGCTGTGTGTGGG/-TGCAGGGAAAGA		252	1	0	0.002	0.004	0.000
MPJ6_2C19_005[a]				Enhancer	15268444	−2545	CTGTGTGTGGGTG/TCAGGGAAAGAGG		252	1	0	0.002	0.004	0.000
MPJ6_2C19_006[a]				Enhancer	15268444_15268445	−2545_−2544	CTGTGTGTGGGTGC/-AGGGAAAGAGGC		252	1	0	0.002	0.004	0.000
MPJ6_2C19_007[a]				Enhancer	15268949	−2040	TAAAGAGAGCAAC/TCAAGCTTATCTT		241	12	0	0.024	0.017	0.030
MPJ6_2C19_008		rs11568732	16)	Promoter	15270100	−889	CAGAATAACTAAT/GGTTTGGAAGTTG		174	70	9	0.174	0.193	0.157
MPJ6_2C19_009		rs12248560	20)	Promoter	15270183	−806	CTGTTCTCAAAGC/TATCTCTGATGTA		249	4	0	0.008	0.008	0.007
MPJ6_2C19_010[a]				Promoter	15270211	−778	GATAATGCGCCAC/TGATGGGCATCAG		252	1	0	0.002	0.000	0.004
MPJ6_2C19_011[a]				Promoter	15270212	−777	ATAATGCGCCACG/AATGGGCATCAGA		252	1	0	0.002	0.004	0.000
MPJ6_2C19_012[a]				Promoter	15270460	−529	TTTCATGTTTAGG/CCTGCTGTATTTT		248	5	0	0.010	0.008	0.011
MPJ6_2C19_013[a]				Promoter	15270800	−189	GACGAAGGAGAAC/AAAGACCAAAGGA		252	1	0	0.002	0.000	0.004
MPJ6_2C19_014[a]				Promoter	15270804	−185	AAGGAGAACAAGA/GCCAAAGGACATT		252	1	0	0.002	0.000	0.004
MPJ6_2C19_015		rs4986894	16)	Promoter	15270891	−98	GATTGGCCACTTT/CATCCATCAAAGA		137	97	19	0.267	0.256	0.276
MPJ6_2C19_016		rs17885098	20)	Exon 1	15271087	99	CCCTCCTGGCCCC/TACTCCTCTCCCA	P33P	9	70	174	0.826	0.807	0.843
MPJ6_2C19_017[a]				Exon 1	15271139	151	ATTAAGGATGTCA/GGCAAATCCTTAA	S51G	252	1	0	0.002	0.000	0.004
MPJ6_2C19_018[a]				Intron 1	15271284	IVS1+128	TTGAAAGGCTTTT/AGTTGCCTTTTCC		250	3	0	0.006	0.008	0.004
MPJ6_2C19_019		rs17884832		Intron 1	15283001	IVS1−340	TTCAAATTTGGGT/GCTTCTGTATATT		174	70	9	0.174	0.193	0.157
MPJ6_2C19_020		rs7916649		Intron 1	15283110	IVS1−231	GTTGGTGTGAGGG/ATTAATTGTAATC		76	127	50	0.449	0.458	0.440
MPJ6_2C19_021		rs17878649		Intron 1	15283294	IVS1−47	GCTTAGTAAATGG/AACAAAACAGTGA		193	55	5	0.128	0.139	0.119
MPJ6_2C19_022		rs12769205	20)	Intron 2	15283650	IVS2−23	GATCTCCCTCCTA/GGTTTCGTTTCTC		137	97	19	0.267	0.256	0.276
MPJ6_2C19_023[a]				Exon 3	15283822	481	AGAAAAACCAAGG/CGTGGGTGAACAT	A161P	250	3	0	0.006	0.000	0.011
MPJ6_2C19_024[a]				Intron 3	15283985	IVS3+163	GAATTTGCACATG/ATTTGTGCTGTGT		252	1	0	0.002	0.004	0.000
MPJ6_2C19_025		rs17879992		Intron 3	15284154	IVS3+332	TTTTTCCCATTAT/CCTATTCCAGAAC		174	70	9	0.174	0.193	0.157
MPJ6_2C19_026		rs4986893	10)	Exon 4	15288936	636	AAGCACCCCCTGG/AATCCAGGTAAGG	W212X	193	55	5	0.128	0.139	0.119
MPJ6_2C19_027[a]				Intron 4	15289213	IVS4+271	TTTCTATAAAGTA/GCTTTGGTGACAG		252	1	0	0.002	0.004	0.000
MPJ6_2C19_028		rs7088784		Intron 4	15289899	IVS4−205	GAAATGATTATCA/GTCTTTGATTCTC		174	70	9	0.174	0.193	0.157
MPJ6_2C19_029		rs4244285	9)	Exon 5	15290142	681	TGATTATTTCCCG/AGGAACCCATAAC	splicing defect	137	97	19	0.267	0.256	0.276
MPJ6_2C19_030		rs12571421		Intron 5	15290508	IVS5+228	TAAATATAATCTA/GTCTGAACAATAA		137	97	19	0.267	0.256	0.276
MPJ6_2C19_031			20)	Intron 5	15328666	IVS5−113	TTTTTCTAGTACT/GATACTTTACAGT		230	23	0	0.045	0.055	0.037
MPJ6_2C19_032		rs4417205	20)	Intron 5	15328728	IVS5−51	ATTTACTGTCATC/GAAATATGCTGTT		140	94	19	0.261	0.248	0.272
MPJ6_2C19_033[a]				Intron 5	15328730	IVS5−49	TTACTGTCATCAA/GATATGCTGTTAA		252	1	0	0.002	0.000	0.004
MPJ6_2C19_034[a]				Intron 6	15350910	IVS6−210	CATATATGTGTAC/TAGATTTTTCTTA		251	2	0	0.004	0.000	0.007
MPJ6_2C19_035[a]				Intron 6	15350924	IVS6−196	GATTTTTCTTAAT/AGCCTAGCTTAAG		138	96	19	0.265	0.252	0.276
MPJ6_2C19_036[a]				Intron 6	15351088	IVS6−32	CCATTTCTCTCCT/ATTTCCATCAGTT		252	1	0	0.002	0.000	0.004
MPJ6_2C19_037[a]				Exon 7	15351144	986	AAGAGATTGAACG/ATGTCGTTGGCAG	R329H	252	1	0	0.002	0.000	0.004
MPJ6_2C19_038	IMS-JST111900	rs3758580	20)	Exon 7	15351148	990	GATTGAACGTGTC/TGTTGGCAGAAAC	V330V	138	96	19	0.265	0.252	0.276
MPJ6_2C19_039	IMS-JST111901	rs3758581	3)	Exon 7	15351149	991	ATTGAACGTGTCA/GTTGGCAGAAACC	I331V	0	23	230	0.955	0.945	0.963
MPJ6_2C19_040[a]				Exon 7	15351236	1078	CAGAGATACATCG/AACCTCATCCCCA	D360N	252	1	0	0.002	0.000	0.004
MPJ6_2C19_041[a]				Exon 7	15351277	1119	AGTGACCTGTGAC/TGTTAAATTCAGA	D373D	252	1	0	0.002	0.000	0.004
MPJ6_2C19_042[a]				Intron 7	15351391	IVS7+84	TCTACCATCACTG/AGGTGAGAGAAGT		252	1	0	0.002	0.004	0.000
MPJ6_2C19_043		rs17882222		Intron 7	15357999	IVS7−201	TCCTGATTGTGGG/ACATTTTAGCAAG		241	12	0	0.024	0.017	0.030
MPJ6_2C19_044[a]				Intron 7	15358026	IVS7−174	TATTGTCACTGGC/TCTTAAGCTCATG		246	7	0	0.014	0.017	0.030
MPJ6_2C19_045		rs4917623		Intron 7	15358094	IVS7−106	TCTTTGGAATGGT/CGTTTCATCATCT		56	126	71	0.530	0.529	0.530
MPJ6_2C19_046		rs17886522	20)	Exon 8	15358301	1251	GGATGAAGGTGGA/CAATTTTAAGAAA	G417G	193	55	5	0.128	0.139	0.119
MPJ6_2C19_047[a]				Intron 8	15358405	IVS8+64	GATCAGTTGGAAC/TTTACATGTGCCT		252	1	0	0.002	0.004	0.000
MPJ6_2C19_048		rs12268020		Intron 8	15360897	IVS8−119	ATCTACTCATCCC/TTCCTATGATTCA		250	3	0	0.006	0.004	0.007

[a]Novel variations detected in this study.

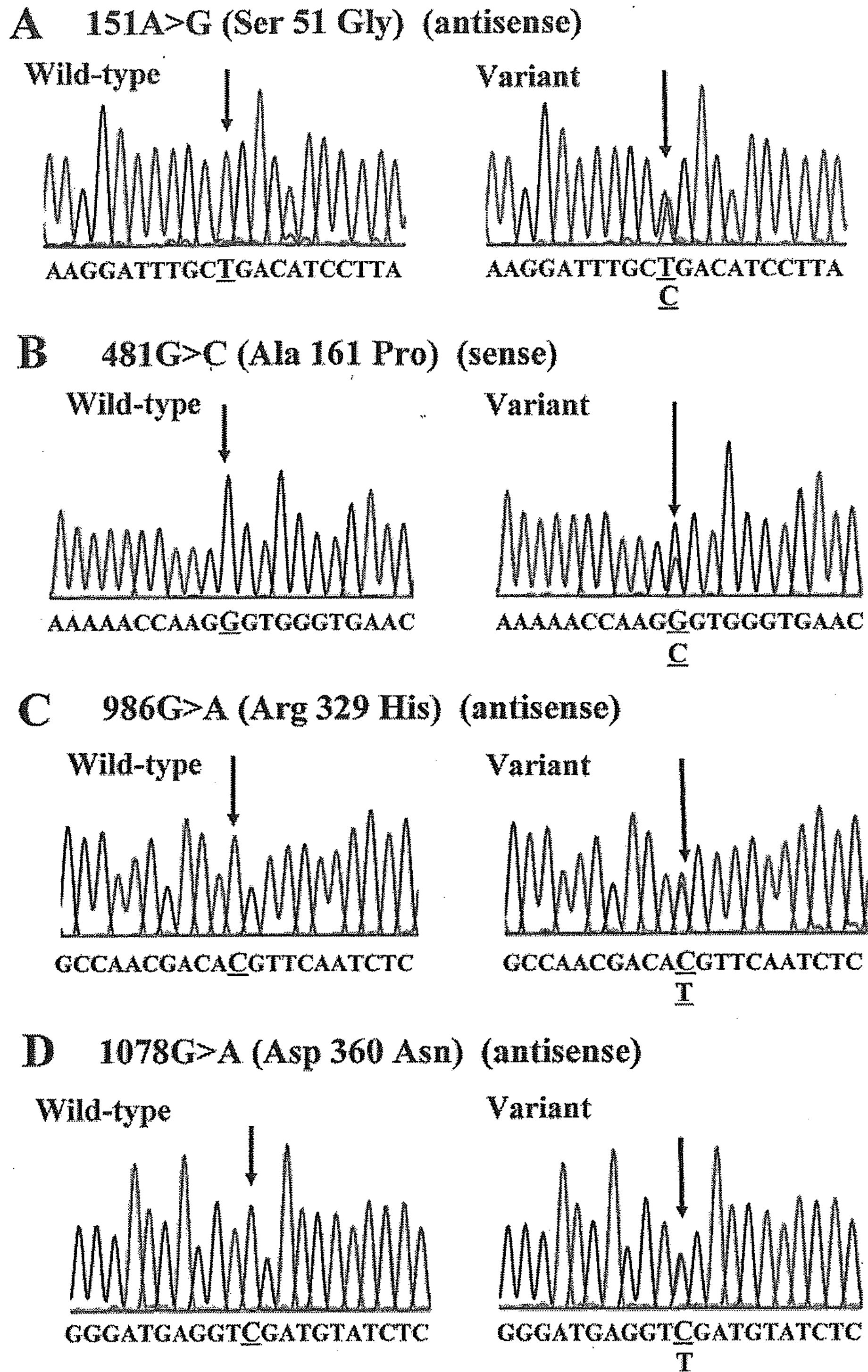

Fig. 1. Four novel nonsynonymous variations of human *CYP2C19*. (A) MPJ6_2C19_017 (wild-type, 151A/A; variant, 151A/G). (B) MPJ6_2C19_023 (wild-type, 481G/G; variant, 481G/C). (C) MPJ6_2C19_037 (wild-type, 986G/G; variant, 986G/A). (D) MPJ6_2C19_040 (wild-type, 1078G/G; variant, 1078G/A). Arrows indicate the positions of the nucleotide changes.

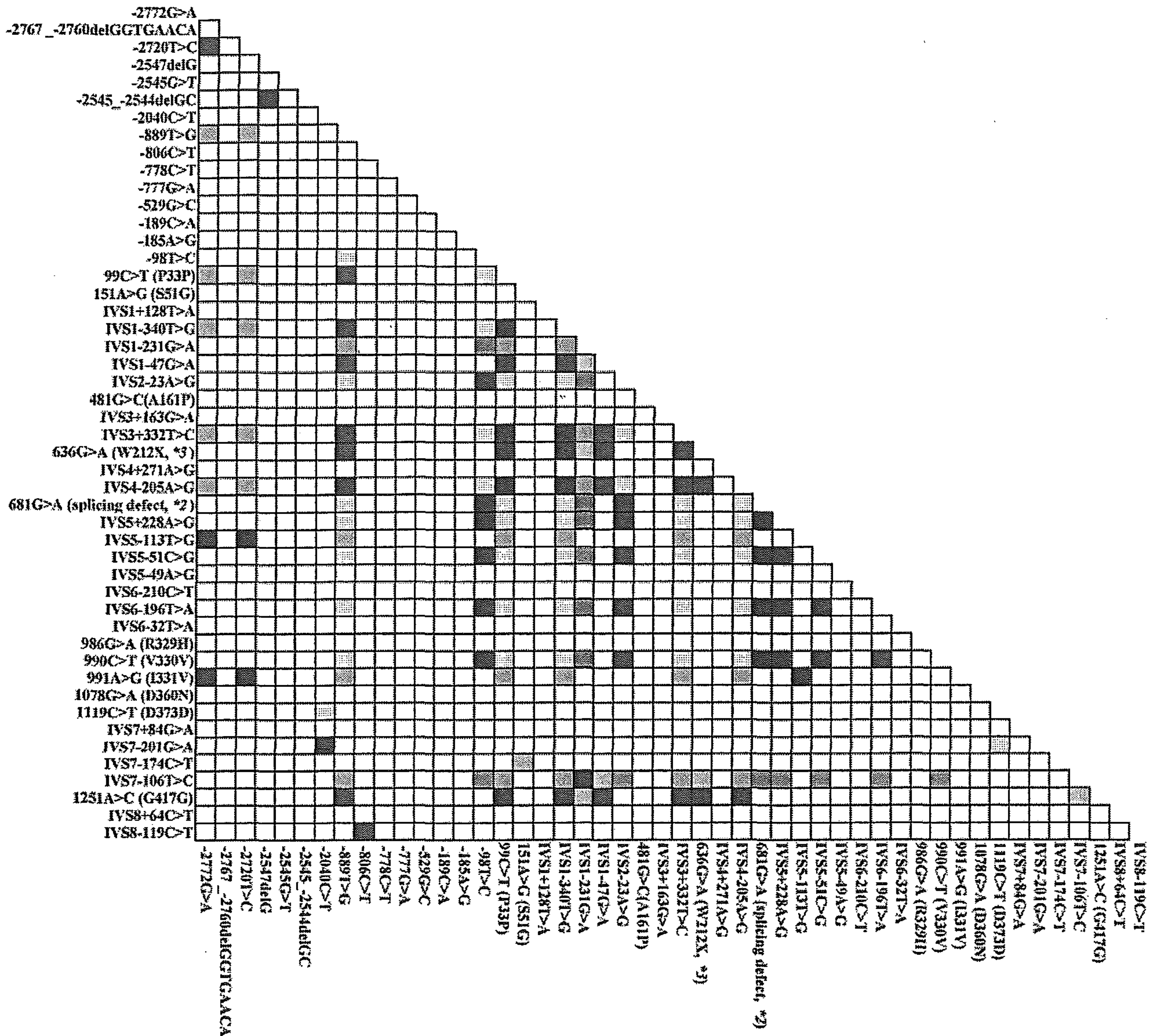

Fig. 2. Linkage disequilibrium (LD) analysis of *CYP2C19* genetic variations.
Pairwise LD is depicted using the r^2 values (from 0 to 1) by a 10-graded gray color. The denser color represents the higher linkage.

conserved in the human CYP2C subfamily.[19] In particular, D360 is located in the substrate recognition site 5.[19] It is possible that the substitution from an acidic (Asp) to a rather neutral (Asn) amino acid might affect the binding of substrates or catalytic activity of the enzyme. The other variations, S51, A161, and R329 are located in the A-helix, in the loop between the D-helix and the E-helix, and in the J-helix, respectively. Further functional analysis should be pursued for these four variations. Moreover, it is necessary to evaluate the real frequencies of the very rare variations found in only one subject (frequency: 0.002).

The two known nonsynonymous SNPs, 681G>A (splicing defect, **2* allele) and 636G>A (W212X; **3* allele) were detected at 0.267 and 0.128 frequencies, respectively. These frequencies were comparable to those of the Japanese in the previous reports.[8,11] The variation 991A>G (I331V, **1B* and **1C* alleles) was found with a 0.955 frequency, indicating that this SNP is rather common in the Japanese population. Other nonsynonymous variations including *CYP2C19*4, *5* and **16* were not detected in this study. Thus, our results confirmed that, except for I331V which has no functional significance, nonsynonymous variations other than *CYP2C19*2* and **3* were very rare in the Japanese population.

Seven and five novel variations were detected in the enhancer and promoter regions, respectively. No variation was found in the reported regions as the binding sites for the constitutive androstane receptor and the glucocorticoid receptor.[17] However, −529G>C was present within the potential negative element (−650 to −453 bases from the translational start site) in the promoter region.[16] Transcriptional activity with this variation needs to be examined in the future.

Using the detected variations, linkage disequilibrium (LD) was analyzed (**Fig. 2**). Perfect linkage ($r^2 = 1$) was observed among −2772G>A, −2720T>C, IVS5-113T>G, and 991A>G (I331V), between −2547delG and −2545_-2544delGC, and between −2040C>T and IVS7-201G>A. Strong LDs were observed among −98T>C, IVS2-23A>G, 681G>A (splicing defect, *2 allele), IVS5+228A>G, IVS5-51C>G, IVS6-196T>A, and 990C>T (V330V) ($r^2 \geq 0.96$), between IVS1-231G>A and IVS7-106T>C ($r^2 = 0.90$), between −806C>T and IVS8-119C>T ($r^2 = 0.75$), and among −889T>G, 99C>T (P33P), IVS1-340T>G, IVS1-47G>A, IVS3+332T>C, 636G>A (W212X, *3 allele), IVS4-205A>G, and 1251A>C (G417G) ($r^2 \geq 0.70$). The r^2 values were below 0.45 for the other pairs of variations. Collectively, the LD was observed throughout the *CYP2C19* gene, suggesting that one LD block covers the entire analyzed region spanning approximately 90 kb. Thus, the *CYP2C19* haplotypes were analyzed as one block.

Haplotype analysis was then performed (**Table 3**). The haplotypes registered to the CYP Allele Nomenclature Committee are shown with the numbers (*18 to *21). The other haplotypes detected in this study are shown as the numbers plus small alphabetical letters. Several haplotypes were first unambiguously assigned by homozygous variations at all sites (*1d, *2c, and *3b) or a heterozygous variation at only one site (*1g, *1h, *1k, *1l, *1m, *1n, and *18). Separately, the diplotype configuration (a combination of haplotypes) for each subject was estimated by LDSUPPORT software. The additionally inferred haplotypes were six *1 subtype (*1e, *1f, *1j, *1p to *1r), eight *2 subtypes (*2d to *2l), four *3 subtypes (*3c to *3f), *19, *20, and *21. The determined/inferred haplotypes were summarized in **Table 3**. As for *20, cloning of the amplified genomic DNA and subsequent sequencing confirmed that 1078G>A (D360N) was linked to the *3b haplotype. The three subjects with heterozygous 481G>C (A161P) had heterozygous 681G>A (*2 allele) concurrently, and the haplotype harboring 481G>C (A161P) was inferred as a subtype of *2c haplotype (designated as *21). The most frequent haplotype was *1d (frequency: 0.492), followed by *2c (0.241), *3b (0.115), *1e (0.043), *1f (0.022), and *1g (0.014). The frequencies of the other haplotypes were less than 0.01.

Table 3. Haplotypes of *CYP2C19* in Japanese population

Nucleotide changes[a] (columns, left to right) and Amino Acid Change:

Nucleotide changes[a]	Amino Acid Change
−2772 G>A	
−2567_−2560 del GCTGAACA	
−2720 T>C	
−2547 delG	
−2548 G>T	
−2545_−2544 delGC	
−2040 C>T	
−889 T>G	
−806 C>T	
−778 C>T	
−777 G>A	
−529 G>C	
−189 C>A	
−183 A>G	
−98 T>C	
99 C>T	P33P
151 A>G	S51G
IVS1+128 T>A	
IVS1-340 T>G	
IVS1-231 G>A	
IVS1-47 G>A	
IVS2-23 A>G	
481 G>C	A161P
IVS3+163 G>A	
IVS3+332 T>C	
636 G>A	W212X
IVS4+77 A>G	
IVS4-205 A>G	
681 G>A	splicing defect
IVS5+228 A>G	
IVS5-113 T>G	
IVS5-51 C>G	
IVS5-40 A>G	
IVS6-210 C>T	
IVS6-196 T>A	
IVS6-32 T>A	
986 G>A	R329H
990 C>T	V330V
991 A>G	I331V
1078 G>A	D360N
1119 C>T	D373D
IVS7+63 G>A	
IVS7-201 G>A	
IVS7-174 G>T	
IVS7-106 T>C	
1251 A>C	G417G
IVS8+64 C>T	
IVS8-119 C>T	

Haplotypes[b,c,d]		Frequency	
*1	*1d	0.492	0.601
	*1e	0.043	
	*1f	0.022	
	*1g	0.014	
	*1h	0.008	
	*1j	0.006	
	*1k	0.004	
	*1l	0.002	
	*1m	0.002	
	*1n	0.002	
	*1p?	0.002	
	*1q?	0.002	
	*1r?	0.002	
*2	*2c	0.241	0.261
	*2d	0.004	
	*2e	0.004	
	*2f?	0.002	
	*2g?	0.002	
	*2h?	0.002	
	*2i?	0.002	
	*2k?	0.002	
	*2l?	0.002	
*3	*3b	0.115	0.126
	*3c	0.006	
	*3d?	0.002	
	*3e?	0.002	
	*3f?	0.002	
*18	*18	0.002	0.002
*19	*19?	0.002	0.002
*20	*20	0.002	0.002
*21	*21	0.006	0.006

[a]A of the translational start codon of *CYP2C19* is numbered 1. NT_030059.12 was used as the reference sequence.
[b]Major allele, white; minor allele, gray
[c]The haplotypes are described as numbers (plus small alphabetical letters).
[d]The haplotypes inferred in only one subject are described with haplotype names and a question mark.

In conclusion, 48 genetic variations, including 27 novel ones, were detected in *CYP2C19* in a Japanese population. Using the detected variations, 31 haplotypes were determined and/or inferred. Our results would provide fundamental and useful information for genotyping *CYP2C19* in the Japanese and probably other Asian populations.

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