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A Symposium on International Health Policy and Medical Waste Management Research in Asian Region

Haruyo Nakamura, Moazzam Ali, Yoshihisa Shirayama, Chushi Kuroiwa*

Keywords: Health care waste, Asian region, 3Rs, Infectious disease

On September 10, 2008, at the University of Tokyo, researchers from China, Laos, Mongolia, Pakistan, Thailand, and Japan exchanged results and shared experiences at the symposium on "The International Health Policy and Medical Waste Management Research in Asian Region". This three-year research was supported by the Ministry of Environment, Japan, highlighting 3Rs (Reduce, Reuse, Recycle) initiative toward the society without wastes, which was expressed by then Japanese Prime-Minister at G8 summit in 2004.

Health care waste (HCW) management is drawing the global attention due to its hazardous nature and the potential to jeopardize not only the health of the patients but also the health workers and the community at large. It is known that inappropriate health care waste management causes infectious diseases, such as hepatitis B, C, and HIV/AIDS. Another relevant and important aspect is the inappropriate utilization of the small-scale incinerators at temperatures below 800°C in developing countries, as they are reported to produce dioxins, furans or other toxic pollutions while burning the wastes.

The introduction of sophisticated disposable equipment has even increased the pressure on already frail health care waste management systems in many developing countries. According to Dr. Chushi Kuroiwa, waste volume of auto-disable (AD) syringe, which was introduced in the pilot measles mass campaign in Laos, would be 200 times as much as those of sterilized syringes. Since the infrastructure for proper disposal of syringes waste is insufficient and inappropriate, consequently syringes are burnt improperly and thus posing the health workers and the communities at increased risk for injuries. He also briefly talked about the new needle removal machine, which has recently got patent in China. The machine is user friendly and of use in resource poor settings and will help the health care providers in achieving safer working environment.

Dr. Alongkone Phengsavanh from Laos reported that although segregations were relatively done well at major hospitals in Vientiane Capital, only 39% of health workers at the biggest hospital knew how the waste should be separated. In northern part of Laos in Luang Namtha province, Dr. Yoshihisa Shirayama identified problems at each step of HCW management in health facilities, and shared that in the survey, 80% of the health workers acknowledged and felt the need that their HCW management system needs improvement. Ms. Yin-Ju Chen presented a detailed report on the adverse affects on the health of scavengers at the final landfill site in Vientiane Capital, Laos.

Dr. Xu Lingzhong, head of the School of Public Health, Shandong University, highlighted that issue of
urban-rural disparity in HCW management in China. Ms. Ruoyan Gai further the discussion by sharing results of her research pointing to differences in hospital waste management at different levels of hospital (such as tertiary, primary care levels). Ms. Zhang Zhuo reported positive impact of education intervention program on blood-born pathogens and injuries among health workers.

Waste-management infrastructure is not sufficient in many developing countries. In Mongolia, Dr. Budbazar Enkhtuya, head of the Department of Immunization reported 11.5% of healthcare facilities had small-scale low-temperature incinerators and even the incinerators in the capital city do not meet the WHO safety requirements. Dr. Hiransuthikul from Chulalongkorn University pointed out the main issues facing HCW management in Thailand. He also reported only 22 out of 859 incinerators run at the recommended temperatures i.e. above 800°C in Thailand.

Dr. Moazzam Ali highlighted issues of health care workers safety and mentioned that the prevalence of needle stick and sharp injury was very high in Mongolian hospitals and majority of injuries occurred among nurses, followed by housekeepers. In majority of cases the common cause of injury was disposable syringe and most injuries occurred during recapping, opening of ampoule or vial and improper disposal of syringes. He also reported malfunction of HCW management in Pakistan hospitals and urged that regular trainings can create awareness and minimize the problem, as the injury incidence was clearly less among the trained health workers.

Dr. Masamichi Kinomoto, head of Biomedical Science Association, Tokyo, stressed the necessity of behavior change among health workers to bring sanitary and safe environment in healthcare facilities. He also highlighted the importance of education on HCW, which should preferably be initiated especially at elementary school level for children.

The presentations were followed by panel discussion on current issues in improving health care waste system in hospitals. It was an interactive session where diverse questions on the important issues were posed to the panelists by the participants. In the end Dr. Chushi Kuroiwa summarized the main issues and thoughts from the symposium and finally thanked the participants for their keen and enthusiastic participation. The symposium ended with a note to further the continued collaboration and research sharing in order to improve the HCW management in the participant countries in moving toward waste free societies.

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- International Health Policy and Medical Waste Management Research in Asian Region (Chushi Kuroiwa, The University of Tokyo, Japan)
- Medical wastes management in Japan (Masamichi Kinomoto, Biomedical Science Association, Tokyo, Japan)
- Hospital Medical Waste Management in Shandong Province, China (Xu Lingzhong, Shandong University, China)
- Health care waste management in Mongolia (Budbazar Enkhtuya, CCD, Mongolia)
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- Vulnerability of Chinese Medical Waste (MW) Management system (Zhang Zhuo, The University of Tokyo, Japan)
- Needle stick & sharp injures (NSSI) in health care workers in Ulaanbaatar, Mongolia
- Challenges and issues in hospital solid waste management: case study from Pakistan (Moazzam Ali, The University of Tokyo, Japan)
- Health status of scavengers working at the dumpsite in Vientiane, Lao PDR (Yin-Ju Chen, The University of Tokyo, Japan)
- Medical waste management in Luang-Namtha province in Lao PDR (Yoshihisa Shirayama, The University of Tokyo, Japan)
- Closing remarks (Chushi Kuroiwa)

(*Health Policy and Planning, the University of Tokyo, Tokyo, Japan)*
Asian pharmaceutical researchers gathered at Japan-China Joint Medical Workshop on Drug Discoveries and Therapeutics 2008

Munehiro Nakata¹, Wei Tang²

Keywords: Drug discovery, Therapeutic, Influenza

Japan-China Joint Medical Workshop on Drug Discoveries and Therapeutics 2008 (JCMWDDT 2008) was held at The University of Tokyo, Tokyo, Japan, September 29, 2008. The workshop was started with an announcement by Chairperson from Japan, Dr. Sekimizu (Department of Microbiology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Japan; Editor-in-Chief of Drug Discoveries & Therapeutics, DDT) followed by a series of speech by Chairperson from China, Dr. Wenfang Xu (School of Pharmaceutical Sciences, Shandong University, Shandong, China; Editor in China Office of DDT), Dr. Norio Matsuki (The University of Tokyo, Japan; Editor of DDT), and Dr. Guanhua Du (Chinese Academy of Medical Science, China; Editor of DDT).

JCMWDDT has been firstly held on May of last year at Shandong University, Shandong, China, to promote research exchange in the field of drug discovery and therapeutic between Japan and China, which is mainly organized by editorial members of Drug Discoveries & Therapeutics (http://www.ddtjournal.com/home), a sister journal of BioScience Trends. JCMWDDT of this year is the second workshop and especially focuses on novel development and technological innovation in anti-influenza virus agents. Annual outbreak of avian influenza in Asian countries including China and Japan spread fears of a mutation of the virus followed by a pandemic in human beings. Thus, it is crucially important for Asian countries to work together to control the infection.

The first lecturer, Dr. Xu, presented his advanced study entitled 'Design, synthesis and preliminary activity assay of influenza virus neuraminidase inhibitors' and showed a predictive structure-based drug design using a consistent QSAR model and a discovery of a novel series of lead compounds to inhibit influenza neuraminidase. Next, Dr. Sekimizu presented his creative study entitled 'Infection disease models with silkworms to evaluate the therapeutic effects of drug candidates' and stated the outstanding availability of silkworms in drug discovery.

The workshop is held for three days from September 29 to October 1 and 59 titles are going to be presented in 6 specialized sessions and a poster session (Drug Discov Ther 2008; 2, Suppl). It is expected that JCMWDDT 2008 would provide opportunities to re-emphasize the crucial position of medicinal chemistry to conquer influenza and create an environment for cooperative researches among Asian countries. (reported on September 29)

Main program

Session I. Research Advances in Drug Discoveries and Therapeutics

- Design, synthesis and preliminary activity assay of influenza virus neuraminidase inhibitors by Wenfang Xu (Shandong University, China)
- Infection disease models with silkworms to evaluate the therapeutic effects of drug candidates by Kazuhisa Sekimizu (The University of Tokyo, Japan)
- Japan’s governmental approaches to facilitate drug development process by Makoto Shimoaraiso (Ministry of Foreign Affairs of Japan, Japan)
- Effective detection of the epidermal growth factor receptor
mutation by the peptide nucleic acid-locked nucleic acid
PCR Clamp by Sakuo Hoshi (The University of Tokyo Hospital, Japan)

• Design and synthesis of p53-MDM2 binding inhibitors by Yongzhou Hu (Zhejiang University, China)

Session II. Drug Synthesis/Clinical Therapeutics

• Pharmacogenomics-based clinical studies using a novel fully-automated genotyping system by Setsuo Havegawa (Sekino Clinical Pharmacology Clinic, Japan)

• Synthesis and biological evaluation of pentacyclic triterpenes as anti-tumor agents by Hongbin Sun (China Pharmaceutical University, China)

• Drug discovery and therapeutics using silkworm as experimental animal by Yasuyuki Ogata (The University of Tokyo, Japan)

• Novel selective estrogen receptor modulators (SERMs) with unusual structure and biological activities by Haibing Zhou (Wuhan University, China)

Session III. Medicinal Chemistry/Natural Products

• Synthesis and properties of isonucleosides incorporated oligonucleotides by Zhenjun Yang (Peking University, China)

• Isolation of antivirus compounds from plant resources using silkworm bioassay by Yutaka Orihara (The University of Tokyo, Japan)

• Synthesis and structural modification of tasiamide and the effect of these modifications on in vitro antiviral activity by Yingxia Li (Ocean University of China, China)

• Spirohexalines A and B, novel undecaprenyl pyrophosphate inhibitors produced by Penicillium sp. FKI-3368 by Junji Inokoshi (Kitasato University, Japan)

• Nosokomycins, novel anti-MRSA antibiotics, produced by Streptomyces sp. K04-0144 by OR. Uchida (Kitasato University, Japan)

• In vivo screening for antimicrobial activity of Thai Herbal Medicines using silkworm model by Santad Chanprapaph (Chulalongkorn University, Thailand)

• Novel electrochemical sensor of nitric oxide for screening anti-aging Traditional Chinese Medicine by Zilin Chen (Wuhan University, China)

• Polysaccharide from green tea purified by silkworm muscle contraction assay induces innate immunity by increasing the expression of various inflammatory cytokine mRNA in human leukocytes by Saphala Dhital (The University of Tokyo, Japan)

Session IV. Anti-influenza Drugs

• Structure-activity relationship of flavonoids as influenza virus neuraminidase inhibitors and their in vitro anti-viral activities by Guanghua Du (Chinese Academy of Medical Sciences and Peking Union Medical College, China)

• Mechanisms and consequences of phagocytosis of influenza virus-infected cells by Yoshinobu Nakanishi (Kanazawa University, Japan)

• Nuclear export inhibitors; a possible target for novel anti-influenza viral drugs by Ken Watanabe (Nagasaki University, Japan)

• Catalytic asymmetric synthesis of oseltamivir phosphate directing toward its stable worldwide supply by Motomu Kanai (The University of Tokyo, Japan)

• Clinical effects of probiotic bifidobacterium in the prevention of influenza virus infections and allergic diseases by Jin-zhong Xiao (Morinaga Milk Industry Co., Ltd., Japan)

• Production of anti-influenza PR8-scFv using a phage display by Normaiza Zamri (Tokai University, Japan)

Session V. Anti-infection/Antiviral Drugs

• Emerging infectious diseases and anti-viral drugs: Urgent need to develop effective drugs which cause less resistant virus by Nobuyuki Kobayashi (Nagasaki University, Japan)

• Design, synthesis and antiviral evaluation of novel heterocyclic compounds as HIV-1 NNRTIs by Xinyong Liu (Shandong University, China)

• Antiviral drug screening from microbial products by Esaka Tsuji (Astellas Pharma Inc., Japan)

• Viral factors that determine the natural course of chronic hepatitis B viral infection by Hiroshi Totsuyanagi (The University of Tokyo, Japan)

• Effect of androgapholide derivatives having α-glucosidase inhibition, on HBsAg, HBeAg secretion in HepG2 2.2.15 cells by Hongmin Liu (Zhengzhou University, China)

• Current and future antiviral therapy for influenza by Hideki Asanuma (Tokai University, Japan)

• Establishment of an HIV-based pseudotyping system as a safe model for screening inhibitors on bird flu H5N1 entry by Ying Guo (Peking Union Medical College Chinese Academy of Medical Sciences, China)

• Strategy of discovery for novel antibiotics using silkworm infection model by Hiroshi Hamamoto (The University of Tokyo, Japan)

• Potent neuraminidase inhibitors and anti-inflammatory substances from Chaenomelis speciosa by Li Zhang (Chinese Academy of Medical Sciences and Peking Union Medical College, China)

• High-throughput screening assay for hepatitis C virus helicase inhibitors using fluorescence-quenching phenomenon by Hidenori Tani (Waseda University and National Institute of Advanced Industrial Science and Technology, Japan)

Session VI. Biochemistry/Molecular Biology/Pharmacology

• A novel conjugate of low-molecular-weight heparin and Cu,Zn-superoxide dismutase: Study on its mechanism in preventing brain reperfusion injury after ischemia in gerbils by Fengshan Wang (Shandong University, China)

• A novel gene fudoh in SCCmec region regulates the colony spreading ability and virulence in Staphylococcus aureus by Chikara Kaito (The University of Tokyo, Japan)

• Water soluble fluorescent boronic acid sensors for tumor cell-surface saccharide by Hao Fang (Shandong University, China)

• Molecular characterization of the biosynthetic enzyme for the biotechnological production of tetrahydrocannabinol, the active constituent of marijuana by Futoshi Taura (Kyushu University, Japan)

• Galloyl cyclic-imide derivative CH1104I inhibits tumor invasion via suppressing matrix metalloproteinase activity by Xianjun Qu (Shandong University, China)

• Neuroprotection by inhibition of GAPDH-MAO B mediated cell death induced by ethanol by Xiao-Ming Ou (University of Mississippi Medical Center, USA)

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Recent advances in research on P-glycoprotein inhibitors

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Summary
The ability of cancer cells to experience intrinsic or acquired resistance to a broad spectrum of structurally and functionally unrelated chemotherapeutic agents, termed multidrug resistance (MDR), is the most common cause of chemotherapy failure. Research has firmly established that most tumors developing MDR are often associated with the over-expression of the permeability-glycoprotein (P-gp), the most extensively characterized of the drug efflux pumps. The development of P-gp inhibitors is acknowledged as a viable means of reversing this MDR phenotype and has received considerable attention throughout the past two decades. However, most P-gp inhibitors identified to date have demonstrated limited clinical success due to limitations in potency and specificity. This paper reviews the most recent discoveries relating to the medicinal chemistry of P-gp inhibitors that are presently in development. In light of this information, this paper seeks to suggest new treatment options for the MDR phenotype.

Keywords: P-glycoprotein, Inhibitor, MDR modulator, Substrate

1. Introduction
Multidrug resistance (MDR) is defined as the simultaneous resistance to various structurally and functionally unrelated drugs, which is believed to be one of the major obstacles of successful cancer chemotherapy (1). One of the best-understood MDR-involved mechanisms is the over-expression of the mdr1 gene-encoded product, P-gp, an ATP-dependent xenobiotic exporter that causes an increased efflux of drugs from cancer cells (2).

P-gp (EC 3.6.3.44, CD243 antigen), a 170-kDa phosphoglycoprotein, contains two highly homologous halves, called NH₂- and COOH-terminal halves, respectively, that are 43% identical in human P-gp (3). Each homologous half contains six transmembrane (TM) helices and one nucleotide-binding domain (NBD) (4). P-gp up-regulation is often found in patients with cancer relapse after chemotherapy and in cultured cells that inevitably become drug resistant after stepwise selection as a result of resistance to chemotherapeutic agents (5).

The classical approach to interfering with abnormal P-gp activity is the use of small molecules as P-gp inhibitors, also called MDR modulators, to block the aberrant function of the P-gp pump, eventually leading to the effective accumulation of cytotoxic drugs in tumor cells (6,7). The development of P-gp inhibitors, including those of natural origin and those chemically synthesized, is acknowledged as a viable means of reversing this MDR phenotype and has received considerable attention throughout the past two decades (8).

Till now, three generations of these molecules have been identified as P-gp inhibitors, which can also be classified into competitive and noncompetitive inhibitors according to their corresponding inhibitory mechanism. Among these inhibitors, many agents that modulate the function of P-gp are able to restore the cytotoxicity of chemotherapeutic drugs to MDR cells in vitro and in experimental drug-resistant tumors in vivo. Unfortunately, only a few compounds have reached
the stage of clinical trials, and none has thus far been cleared for clinical therapeutic use (9).

There are primarily two main reasons for this difficulty: 1) MDR is a complex phenomenon that may arise from several different biochemical or biomolecular mechanisms, with the consequence that inhibition of transporter proteins may, to some degree, be insufficient to completely reverse it; and 2) Despite years of efforts of functionally analyze its structure, the exact physiological role of P-gp has yet to be fully clarified and still appears to be somewhat of a mystery. Moreover, the current failure to achieve effective MDR control in particular requires more potent modulators with proper selectivity and pharmacokinetics in order to avoid unwanted side effects. This is because P-gp not only contributes to antineoplastic resistance by elevating the cellular apoptotic threshold but is also expressed in normal tissues such as the adrenals, gravid uterus, kidney, liver, colon, and capillary endothelial cells in the brain. Consistent with this, the expression of P-gp in these normal tissues serves to prevent the uptake of xenobiotics and prevent exposure of sensitive tissues to xenobiotic agents (10).

Therefore, great efforts are still needed to further identify novel compounds that inhibit abnormal P-gp function in order to reverse the MDR phenotype and sensitize cancer cells to conventional anticancer drugs without unwanted toxicological effects (11).

2. Competitive and noncompetitive P-gp inhibitors

2.1. Competitive inhibitors

Competitive P-gp inhibitors, as substrates for P-gp, compete with cytotoxic agents for transport by the pump, as shown as Figure 1A. If both the drug substrate and the inhibitor have similar affinity, the greater the concentration of inhibitors, the less chance there is for the substrate to enter the active drug-binding sites of P-gp. Conversely, the greater the concentration of substrate, the less efficient is the inhibition of P-gp. However, if the P-gp inhibitor has a relative low affinity for drug-binding sites, a high concentration of the inhibitor is thus required to achieve the anticipated effect, consequently limiting the efflux of xenobiotics and accordingly increasing its intracellular concentration (12).

Many first- and second-generation P-gp inhibitors are just such competitive inhibitors; in other words, they compete as a substrate for P-gp with xenobiotics. In addition, the high degree of similarity in structure, partition coefficient (log P), and membrane interaction between substrates and inhibitors has caused great difficulty in defining their molecular properties. Thus, these similarities should be avoided when designing a potent P-gp inhibitor.

2.2. Noncompetitive inhibitors

In contrast to their competitive counterparts, noncompetitive inhibitors of the P-gp transporter do not compete with the substrate for active binding sites in the pocket, as shown in Figure 1B. Such inhibitors usually bind to a different region of the protein in comparison to the substrates, and in doing so they can induce a conformational change in the protein via a cross-linking pattern that is affected by the structure of the drug substrate (or what is generally termed an induced-fit mechanism) so that the active site is no longer recognizable to the hydrophobic substrates. This thus prevents ATP hydrolysis and transportation of the anticancer drugs out of the cell, resulting in an increased intracellular concentration. In simple terms, increasing the amount of substrates in cell will have no effect on

![Figure 1](https://www.biosciencetrends.com)
the level of inhibitor (1). Third-generation inhibitors of P-gp can bind with high affinity to the transporter pump but are not themselves substrates, so they thus fall under the category of noncompetitive inhibitors.

3. The active drug-binding sites of P-gp

Exploration of the active drug-binding sites of P-gp has always been a major and interesting aspect of the P-gp issue. It is generally accepted that there are at least three drug/substrate binding sites and one allosteric site of P-gp; these are independent but interact with each other to perform the transporter function of P-gp (13). Many reported P-gp inhibitors primarily interact reversibly with one or more of the three presumed drug/substrate-binding sites and thus are competitive inhibitors (14).

Studies with thiol-reactive substrate analogs of P-gp and cysteine mutants have shown the drug-binding site is a "funnel-shaped pocket" surrounded by 12 TM segments; the site is narrow on the cytoplasmic side, at least 9-15Å wide in the middle, and wider at the extracellular side; it allows only stipiamide dimers greater than 11Å but less than 35Å in length (15). This drug-binding pocket might be large enough to accommodate more than one substrate at the same time by using an organic combination of amino acid residues from different TM regions to form a specific drug-binding site for a particular drug. Thus, different substrates might occupy different binding sites (16).

4. Three generations of P-gp inhibitors

Over the past two decades, P-gp inhibitors have gone through three generations of development, as will be discussed in the following sections.

4.1. First-generation P-gp inhibitors

The term "first generation" implies those drugs that already in clinical use for other pharmacological activities but not specifically developed in order to reverse P-gp-mediated MDR. Such drugs, however, were occasionally found to possess such ability in clinical trials. Typical examples are the calcium ionic channel blockers verapamil (VRP) and diltiazem (herbesser), the immunosuppressive agent cyclosporin (cyclosporin A), the estrogen receptor antagonist tamoxifen (TAM), and several calmodulin antagonists, which are themselves substrates of the P-gp pump and inhibit P-gp-mediated drug transport in a competitive manner.

Most first-generation inhibitors proved to be effective in vitro, and some were even extremely efficient at MDR reversal. Unfortunately, they were minimally effective, non-specific, and toxic. Due to their mechanism of competitive action and the resulting low binding affinities with transporters, high in vivo serum concentrations of inhibitors were thereby required to obtain sufficient intracellular concentrations of the cytotoxic drugs as well as effective inhibition of P-gp, resulting in, to a large extent, severely unacceptable toxicities for cancer patients. Therefore, these drugs often produced disappointing results in vivo and have limited clinical usefulness mainly due to their severe side effects and toxicity. What is worse, some, e.g. quinine, even proved to specifically beneficial at reversing the overexpression of P-gp when used in the treatment of MDR (17).

In addition, numerous studies have found that many of these inhibitors are substrates both for transporter proteins and other physiological drug transporting enzyme systems, e.g. drug-metabolizing cytochrome P450 isoenzyme 3A4 (CYP3A). Because both CYP3A4 and P-gp are expressed in metabolic-related tissues, such as the intestine and the liver, they may work together to eliminate xenobiotics. Thus, the inhibition of P-gp will interfere with normal metabolism and possibly retard the biotransformation of drugs, leading to unpredictable drug-drug interactions and unexpected pharmacokinetic effects, as well as increased toxicity (18).

4.2. Second-generation P-gp inhibitors

To overcome the limitations of first-generation drugs, MDR modulators with improved potency, specificity, and P-gp-binding affinity were exhaustively examined. Second-generation inhibitors were thus developed and can be divided into two categories. The first, which accounts for the majority of the drugs, is limited to analogues of first-generation drugs, including dextrapamil, dextrignuldipine, trans-flupentixol, cyclosporine A analog valsodar (PSC833), and quinidine analog MS-209. The second category mostly consists of those investigational agents with novel chemical structures, such as S-9788, GF-12918, and VX-710 (biricodar).

These drugs proved to have a better pharmacologic profile with relatively high P-gp inhibitory capacity and fewer toxic side effects. Some, such as PSC833, VX-710, and S9788, have undergone phase I, II, or even III clinical trials, but results have been unsatisfactory (19,20).

Given that these inhibitors still detain intracellular xenobiotics by a competitive approach, together with the fact that many are still substrates for CYP3A4, they present similar issues to those with first-generation P-gp inhibitors. That is, the unpredictable pharmacokinetic interactions between the inhibiting agent and anticancer drugs remained unresolved.

Furthermore, the relatively lower specificity and P-gp affinity of these inhibitors led to many, e.g. valsodar and verapamil, affecting several other ABC transporter family members besides the P-gp transporter, such as MRP1 (multidrug resistance protein 1), another
important ABC transporter protein involved in the cancer cell MDR phenotype (21). Studies by Lawrence et al. revealed that although P-gp and MRP1, two of the most often-studied MDR-related transporters, have pharmacological properties that only partially overlap, they do possess transporter selectivity. Inhibitors that alternatively antagonize P-gp over MRP1 are an optimal choice for effective MDR modulators (22).

Finally, in spite of the acknowledged high correlation between overexpression of P-gp and the MDR phenotype, the localized expression of P-gp has also been detected in normal cells or tissues with a secretory function, including the pancreatic gland, adrenal cortex (AdC), kidney, hepatocytes, epithelial tissue (e.g. intestinal epithelium), and cells that constitute the barrier and metabolic functions in the intestine, brain microvascular endothelia, and blood brain barrier (BBB) (10). The abundant expression of P-gp in these normal tissues or organs suggests that it may play a protective role in absorption, transportation, or secretion of proteins or hormones, distribution or detoxification of xenobiotics, and similar processes (23). Consistent with these facts, inhibition of the overexpression of P-gp might also lessen the ability of normal cells or tissues to endure cytotoxic agents. In other words, this inhibition of nonselective transporters may lead to greater adverse effects of anticancer drugs.

4.3. Third-generation P-gp inhibitors

Given the drawbacks of traditional first- and second P-gp inhibitors, such as targeting interrelated proteins, undesirable intrinsic toxicity, and pharmacokinetic interactions, MDR modulator studies have tended to emphasize trying to overcome these disadvantages. The compounds studied are expected to have specific and potent interaction with the P-gp transporter without inhibiting other ABC family transporters. Recently, the advancement of computational chemistry, chemoinformatics, and molecular pharmacology, together with technologies for combinatorial chemistry, have led to MDR modulator discovery shifting from blind screening to rational drug design. As a consequence, third-generation P-gp inhibitors with novel chemical structures have quickly been developed. These inhibitors were designed with the guidance and elucidation provided by quantitative structure-activity relationship (QSAR) studies, as well as use of noncompetitive mechanisms. Many of the inhibitors thus developed are in the clinical stage and hold promise for future treatment, including VX-710 (biricodar), tetrandrine, FG020326, S-9788, GF-120918 (elacridar), LY-335979 (zosuquidar), tariquidar (XR9576, Xenova), laniquidar (R101933), and ONT-093 (OC144-093) (24). These investigational agents have minimal effect on other members of the ABC transporter family and dramatically reduced the interaction with CYP 3A4 at relevant concentrations (25).

More importantly, the vast majority of the agents tested thus far have caused minimal clinically relevant alterations in the pharmacokinetics of co-administered anticancer drugs in vitro. Such specificity for the P-gp pump minimizes the possibility that the blockade of more than one pump might result in altered bioavailability or excretion of the chemotherapy agents. The advantages of these noncompetitive P-gp inhibitors make them potential MDR reversing agents and offer significant improvements in chemotherapy without the need for chemotherapy dose reductions (5).

5. Newly identified P-gp inhibitors

Despite the strong potency of third-generation P-gp inhibitors in vitro, there is no commercial drug for use in MDR therapy. This is due to disappointing results from either in vivo or preclinical assays. Given the lessons learned from failed trials, novel P-gp inhibitors with high specificity and potency, little effect on metabolic enzymes, and reduced pharmacokinetic effects are in great demand to increase survival for cancer patients. Recently, a number of natural and synthetic inhibitors have thus appeared, expanding MDR research.

5.1. Several predicted pharmacophore models concerning P-gp inhibitors

Identifying molecules that specifically interact with P-gp is pivotal for drug discovery; useful pharmacophore models provide direct and tangible information for rational drug design. Current studies are concentrating on two alternative approaches, pharmacophore models using computational methods and database screening from reported molecules in the literature to predict their affinities as inhibitors in vitro.

Over the last several years, several pharmacophore models concerning P-gp inhibitors have been built through three-dimensional QSAR analysis of various reported P-gp substrates and inhibitors. As a consequence, P-gp inhibitors with potential MDR reversal activity might generally consist of the following physico-chemical characteristics: (a) Liposolubility with hydrophobic centers (e.g. aromatic rings or hydrophobic substituents); (b) Cationic species or a basic center in the physiological pH range; (c) At least two coplanar aromatic rings, and hydrophobic substituted aromatic rings to help activity; (d) Appropriate hydrogen bond acceptors (O or N atom), and/or hydrogen bond donors (OH or NH group) with certain spatial separation (26-29)

In 1997, supplementary information was provided by QSAR studies by Klopmann et al. (30). They found that the introduction of a carboxyl group, quaternary
ammonium salt, and substituent groups like aniline, phenol, and \( \text{N}=\text{CH}-\text{CH}= \) were detrimental to activity, so such substituents should thus be avoided.

In 2002, Garrigues et al. (31) derived two predictive MDR reversal pharmacophore models by means of a molecular modeling approach, suggesting that the azimuthal distribution of hydrophobic and polar elements (rather than chemical motifs) as well as the size of ligands of various MDR modulators affect the patterns of interaction with P-gp. They characterized two different but partially overlapping models and indicated that ligand size affected their ability to bind with P-gp.

At nearly the same time, Ekins et al. (32) used in vitro data associated with inhibition of P-gp function to build catalyst 3D-QSAR models that could qualitatively rank, order, and predict IC\(_{50}\) values for P-gp inhibitors that may modulate one or more P-gp binding sites. As an extension of their work, they further deduced a new pharmacophore to validate their previous results, suggesting that digoxin and vinblastine are likely to bind similar or overlapping P-gp binding sites summarized from inhibition of verapamil binding (33).

In 2006, Crivori et al. (34) used two different computational models with inhibition of cellular molecular descriptors and multivariate analysis to identify potential P-gp substrates and inhibitors. The highly predictive models were capable of predicting correctly the behavior of 72% of an external set of 272 proprietary P-gp-associated compounds. Their results should prove highly useful to medicinal chemists in the search for prospective drug candidates with high success rates and in structure-based virtual screening.

To investigate plausible explanations for the pharmacokinetic profiles of published inhibitors, Chang et al. (35) used both pharmacophore models and database screening to rapidly and accurately confirm the affinity properties (inhibitor or substrate) of tested molecules when binding with P-gp. At the same time, they used biopharmaceutics and drug disposition classification to also provide insight into anticipated drug-drug interactions, which is especially useful in efficiently facilitating screening in vitro in order to avoid compounds with a potential and specific P-gp interaction.

5.2. **Natural inhibitors of P-gp**

Piperine (Figure 2), a major component of black pepper (Piper nigrum Linn) and long pepper (Piper longum Linn) used as a spice and nutritional supplement, is a common dietary constituent and phytochemical. Research has verified that piperine can inhibit the P-gp-mediated transport of digoxin and cyclosporine in the Caco-2 cell monolayers (36). More recently, Han et al. (37) performed in vitro and in vivo assays at dietary levels. They indicated that piperine can affect P-gp function and expression in a manner that is time- and concentration-dependent. Nevertheless, the mechanisms for the piperine-mediated modulation of P-gp expression have yet to be verified. In addition, dietary piperine could affect plasma concentrations of both P-gp and metabolizing enzyme CYP3A4 substrates in humans, so its pharmacokinetic effects are still unclear (36).

Fumagillin (Figure 3) isolated from aspergillus fumigatus is an antibiotic effective against sporidiae to treat nosema disease in bees and fish. Dupuy et al. (38) proved that fumagillin can interfere with P-gp function, revealing that proper optimization of the fumagillin scaffold or derivatization might generate optimal novel fumagillin-derived P-gp inhibitors.

5.3. **Synthetic inhibitors of P-gp**

Recently developed WK series compounds target tetrahydroisoquinolin-ethyl-phenylamine based WK-X-34, WK-X-50, and WK-X-84, as shown in Figure 4. These compounds were found to be very potent, specific, and non-toxic inhibitors of P-gp- and breast cancer resistance protein (BCRP)-mediated MDR in different cell lines using specific in vitro- and in vivo-imaging techniques. They may thus be potential candidates for therapy to treat MDR-resistant tumors.

Within the WK series, WK-X-34 has proven to be the most promising P-gp inhibitor with IC\(_{50}\) values on the order of the nM (39). It has also displayed reduced cellular toxicity and increased potency of both P-gp (IC\(_{50}\) = 82.1 ± 6 nM) and BCRP inhibition (IC\(_{50}\) = 26.5 ± 4.6 µM) in comparison to cyclosporine A, a first-generation P-gp inhibitor with broad-spectrum MDR modulating activity in clinical trials. WK-X-34 does so by inhibiting not only P-gp but also other MDR-associated proteins such as BCRP, MRP1, and lung-resistance protein (LRP) (5,40).

Two synthetic taxane-based compounds (Figure 5), Orataxel (formerly called BAY 59-8862 or IDN-5109)
and tRA 96023 (or SB-RA-31012), have recently emerged as interesting clinical candidates. These candidates could overcome resistance to paclitaxel with broad-spectrum ABC protein (P-gp, MRP1, BCRP) modulating activity and allow oral administration as well. Ortataxel is a cytotoxic taxane that can effectively block its own efflux from P-gp-overexpressing cells by virtue of modulation of P-gp-mediated transport. In contrast, tRA 96023, also can modulate P-gp function but is nontoxic, which may be attributed to the removal of the tubulin-binding side chain at the C-13 position of the taxane scaffold. Such a compound is in preclinical development (4,12).

Pleban et al. (42) demonstrated that the combination of autocorrelation vectors and self-organizing artificial neural networks is an extremely valuable method of identifying P-gp inhibitors with a structurally new backbone. Another approach involving pharmacophore-driven photoaffinity as well as protein homology modeling was used to glean more useful information about P-gp inhibitors. Results indicated that two of the propafenone-type inhibitors of P-gp, AG-690/11972772 together with AN-989/14669159 (Figure 6), were highly active with IC_{50} values below 1 μM (42).

Figure 4. Chemical structures of WK series compounds.

Figure 5. Chemical structures of synthetic taxane-based compounds Ortataxel and tRA 96023.

Figure 6. Chemical structures of propafenone-type inhibitors of P-gp.
Given the fact that the paclitaxel-based taxoid IDN5109 and docetaxel-based IDN5390 are potential clinical candidates to eliminate resistance to paclitaxel, Barboni et al. (43) prepared their corresponding methoxylated analogs 1 and 2, as depicted in Figure 7. They did so in order to improve cytotoxic potency and substantially retain bioavailability. They found that a modification in the form of inserting of a meta-methoxy group in the C-2 benzoate caused a general increase in cytotoxicity and had minimal effect on water solubility. However, the assumption that introduction of a methoxyl group might dramatically effect drug metabolism must still be confirmed.

Similar modifications have also been made by Fong et al. (±)-3′-O,4′-O-dicinnamoyl-cis-khellactone (DCK) is a noncompetitive P-gp inhibitor via derivatization of (±)-praeruptorin A (PA), which is a major component of the extract of Peucedanum praeruptorum Dunn. Since DCK possesses better P-gp-mediated MDR reversal activity than PA itself or even verapamil, the methoxylation of the cinnamoyl groups on DCK was therefore investigated in order to enhance its bioactivity (44). Two novel pyranocoumarins, (±)-3′-O,4′-O-bis(3,4-dimethoxycinnamoyl)-cis-khellactone (DMDCK) and (±)-3′-O,4′-O-bis(4-methoxycinnamoyl)-cis-khellactone (MMDCK), resulted, as shown in Figure 8. They were found to have different P-gp-inhibitory activity. The successful outcome of the modification is the co-existence of 3- and 4-methoxy groups on cinnamoyl (DMDCK), resulting in markedly enhanced activity through a noncompetitive mechanism. To be precise, Fong et al. speculated that the additional 3-methoxy group on cinnamoyl allows DMDCK to interact more efficiently with the P-gp substrate site(s). Meanwhile, the lone existence of the 4-methoxy group on cinnamoyl (MMDCK) reduced activity, and removal of the 4-methoxy group on cinnamoyl (DCK) resulted in moderate activity. A further pharmacophore search with a verapamil-based template also provided supplementary proof that four functional groups of DMDCK could be simultaneously involved in interaction with P-gp whereas for DCK or MMDCK only three groups were involved.

Given that the sigma-2 receptor agonist PB28 demonstrates good P-gp inhibitory activity with an EC₅₀ value of 0.55 μM, Colabufo et al. (7) recently developed its analogs as lead compound 3. They consequently prepared the corresponding series of biphenyl and 2-naphthyl isoquinoline derivatives, as shown in Figure 9.

SAR studies showed that in the biphenyl series (4–6), 5a (X=CH₂, R=OH) provided the best results with an EC₅₀ value of 0.05 μM, which is superior to the lead compound (EC₅₀ = 0.55 μM), while its amide derivative 5b was found to be less active with an EC₅₀ value of 3.5 μM. This suggests the importance of basicity and the apparent unimportance of the presence of –OH or –OCH₃ substituents. In the 2-naphthyl series, both basicity and the presence of an H-bond

![Figure 7](image1.png)

**Figure 7.** Chemical structures of methoxylated analogs derived from IDN5109 and IDN5390.

![Figure 8](image2.png)

**Figure 8.** Chemical structures of methoxylated analogs DMDCK and MMDCK derived from DCK.

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donor or acceptor seem to be of negligible importance. In addition, Colabufo et al. (7) also proposed that lipophilicity did not affect the P-gp inhibitory activity among the compounds.

6. Conclusions and perspectives

During the past two decades, significant progress has been made in understanding the pharmacological and physiological role of P-gp. Concomitant use of P-gp inhibitors is hopefully an effective and safe way to perform further preclinical and clinical investigations with the hope of providing new treatment options to overcome the MDR phenotype.

However, the complex mechanisms of tumor MDR in the body makes the effective use of P-gp-targeted MDR reversing agents/modulators a difficult task. This is largely attributable to, in spite of years of efforts, uncertainty about MDR-associated mechanisms and interaction of inhibitors/substrates with P-gp as well as lack of agreement in those areas (45,46). The formation of the MDR phenotype is a complex and multi-factor process, so focusing attention on certain aspects would prove unfruitful. In addition, this is why, to a large extent, many P-gp inhibitors have been identified but failed to lead to MDR modulating drugs (47).

For instance, one fascinating mystery of P-gp is how its complex protein transport system can recognize and transport a wide variety of structurally unrelated compounds and perform different functions. Requirements for potential clinical candidates are basically that they are novel MDR modulators with broad-spectrum modulation, they lack significant toxicity, and they lack significant pharmacokinetic interactions with cytotoxic drugs. The potency of inhibition of metabolizing enzymes (e.g. CYP3A4) does not necessarily predict a drug’s potency of inhibition for P-gp and vice versa, despite the fact that many molecules interact with CYP3A4 and P-gp to a similar extent (48).

Although P-gp inhibition seems to be a complex and difficult task, a large amount of work is still needed to optimize this strategy. The continued development of detection technology, e.g. computational virtue screening techniques, 3-D QSAR studies, molecular pharmacology, and chemoinformatics, together with the technologies of chemogenomics, will deepen the understanding of P-gp’s structure and efflux mechanisms. Accordingly, this should offer the opportunity for novel therapeutically effective P-gp inhibitor candidates.

Acknowledgements

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**Summary**

Complete loss of function in the WRN: RecQ3 DNA/RNA helicase gene causes Werner Syndrome (WS). WS patients with genetic instability manifest an early onset of age-related diseases including diabetes mellitus (DM), osteoporosis, atherosclerosis, and malignancy as well as early death. In 1,420 patients, WS was reported to be associated with chromosomal abnormality syndrome and other genetic diseases including Klinefelter syndrome in 2 patients, retinitis pigmentosa in 3, Wilson’s disease in 1, xeroderma pigmentosum in 3, and porokeratosis Mibelli in 1. These clinical findings may support the concept of genetic instability in WS.

**Keywords:** Aging, Genetic instability, Mutation, Werner syndrome, Xeroderma pigmentosum

1. **Introduction**

Half a century ago, Dr. Alex Comfort encouraged medical researchers to look for evidence of chromosomal abnormalities in many forms of constitutional disorders, the most important of which was Werner syndrome (WS: MIM#27770) (1). However, his theory faltered since normal chromosomes were found in WS (2-5). WS, the gene located at chromosome 8p11-12 (6) and caused by a recessively mutated WRN, is characterized by a variety of clinical manifestations mimicking features of advanced aging and thus may represent a typical progeroid syndrome (7). Wrn is a member of the RecQ helicase gene family (RecQ3) and may interact with a variety of DNA/RNA metabolism enzymes during repair, transcription, translation, recombination, replication, and chromosome segregation in the nucleus (8). Thus, actively proliferating cells may be affected by WRN dysfunction. Theories on the function of RecQ helicases and in vitro studies using WS fibroblasts and peripheral blood cells have suggested genomic instability in WS cells (8-12). Patients with WS do not usually have apparent abnormalities before their teenage growth spurt, but they typically display hierarchical deterioration of a variety of connective tissue systems resulting in physical symptoms such as gray hair, alopecia, skin atrophy, skin sclerosis, skin hyper/hypo-pigmentation, vocal cord atrophy, osteoporosis, sarcopenia, bilateral cataracts, metastatic subcutaneous calcification, and atherosclerosis. Connective or supportive tissue may be a source of malignancies (and particularly sarcomas), and adversely affected systems include the endocrine system, resulting in type II diabetes mellitus (DM), hypogonadism, and thyroid disorders, and the metabolic system, resulting in hyperlipidaemia, hyperuricemia and hyaluronuria. Systems affected to a lesser degree include the immune system, resulting in excessive auto-antibody production, impaired cytokine response, and natural killer cell activity, and the nervous system, resulting in cognitive disorders and brain atrophy (5,7,13). Death due to malignancy or atherosclerosis-related conditions such as myocardial infarction typically occurs in the late 40s (13-15). In addition, in vivo mutation as may be associated with genetic instability of WS cells may induce other genetic diseases.

Since the first description of WS by the German family physician Otto Werner in 1904 (16), 1,420 cases of WS have been reported in total worldwide (17). Interestingly, 75% of WS patients are of Japanese
descent (14), which is probably due to the relatively high frequency of consanguineous marriage in rural areas and an extremely high prevalence (1:100) of heterozygosity in the general Japanese population (18,19). Approximately 10 WS cases per year are regularly documented in Japan, while only 3.3 patients per year are reported outside the country. Adjusting roughly for population size, the frequency of WS in Japan is some 150-fold greater than in the rest of the world (Japan's population between 1966 and 2004 was about 113,000,000 with respect to a world population of about 5,000,000,000; that said, case reports are highly encouraged in Japan in comparison to some parts of the world).

Since the cloning of the WRN: RecQ3 helicase gene, the search for mutation-causing mutations in WS has been a matter of scientific/clinical interest (20). The current study looked for chromosomal abnormalities and genetic diseases associated with WS in WS patients.

### 2. Materials and Methods

The first case of WS in Japan was reported in 1917 (21). Here, the clinical manifestations of WS as described in all papers published between 1917 and 2004 were analyzed. WS publications were selected through a citation index (Igaku-Chuo-Zasshi) and bibliographies of each report were extensively examined for additional references. For comparison of Japanese and foreign patients with WS, searches were performed using PubMed. Care was taken to thoroughly identify patient family details, personal histories, authors, institutions, and demographic characteristics to avoid the inclusion of duplicate patient data.

As most patients were diagnosed clinically with WS, diagnoses given by the original authors were carefully re-evaluated based on the presence of the following phenotypes: unusual body habitus, bilateral cataracts, skin sclerosis, painful corns, sarcopenia, metastatic subcutaneous calcification, skin ulcers, DM, and hyperlipidaemia (7,13,14).

### 3. Results and Discussion

A total of 1,070 cases documented in 500 Japanese articles published between 1917 and 2004 were included in the analysis. Outside Japan, 350 cases of WS have been reported. As shown in Table 1, 1 case of WS associated with Klinefelter syndrome was diagnosed by chromosomal testing in Japan (22) and one was similarly diagnosed outside Japan (23). Several WS cases have been diagnosed as pseudo-Klinefelter syndrome because of several clinical similarities such as slender extremities with stocky trunk, gynecomastia, and atrophic testis (24,25). However, chromosomal analysis of those cases indicated that they were normal males (26). The frequency of Klinefelter syndrome was about 1 in 700 live-born males in the general population, and the frequency among WS patients was about the same (27). In addition, one WS case of a patient with an extra chromosome was reported by Tanihara et al. in Japan (28).

In 1 Japanese case and 2 European cases of WS, patients had retinitis pigmentosa (29-31). Retinitis pigmentosa has several types of transmission and an overall frequency of about 1 in 3,700 (32). Thus, an incidence of 2:350-1:1070 among WS patients was probably higher than in the general population.

In 1 Japanese case of WS, the patient had Wilson disease (33). The frequency of Wilson disease was about 1 in 30,000-100,000 livebirths worldwide (32). Of particular interest, 3 female siblings with WS may have the variant form of xeroderma pigmentosum (X-P) (34,35). The frequency of all types of X-P in Japan was about 1 in 40,000, which was higher than in the rest of the world (27). However, both the cases of WS and of X-P were clinically diagnosed 50 years ago.

WS has been classified as a genetic instability syndrome, which includes Bloom syndrome (36,37), Rothmund-Thomson syndrome (38), Cockayne syndrome (37), ataxia telangiectasia (40,41), X-P (39), Fanconi anemia (Alter BP, NCI personal communication), and progeria (20). However, no association of additional chromosomal abnormalities or genetic diseases with genetic instability syndromes was noted except for WS and Bloom syndrome. Machino reported a case of WS with porokeratosis Mibelli, and Takemiya described a case of Bloom syndrome with porokeratosis Mibelli (42,43). The frequency of porokeratosis Mibelli in the general population is not known.

In all of the cases analyzed, WS and additional genetic diseases such as retinitis pigmentosa, Wilson disease, and X-P were clinically determined, which merely suggests that WS has genetic instability when encountered clinically.

Finally, some of the aging-associated phenotypes seen may relate directly to WRN dysfunction. Aging is believed to induce genetic instability leading to cancer (44,45), and thus the complete loss of WRN function may epigenetically and genetically impact.

### Table 1. Mutation causes mutations?

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extrachromosome</td>
<td>43 F; 46/XX, 49/XX; Tanihara 1986</td>
</tr>
<tr>
<td>Retinitis pigmentosa</td>
<td>58M; Tajima 1993</td>
</tr>
<tr>
<td></td>
<td>39M; Valero 1960</td>
</tr>
<tr>
<td></td>
<td>38M; Kleberg 1949</td>
</tr>
<tr>
<td>Wilson disease</td>
<td>55M; Sakai 1990</td>
</tr>
<tr>
<td>Xeroderma pigmentosum</td>
<td>23F; Takahashi 1955</td>
</tr>
<tr>
<td></td>
<td>29F; Takahashi 1955</td>
</tr>
<tr>
<td></td>
<td>29F; Takahashi 1955</td>
</tr>
<tr>
<td>Porokeratosis Mibelli</td>
<td>49M; Machino 1984</td>
</tr>
</tbody>
</table>

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other genes, promoters, or proteins related to aging-associated pathophysiology. It may also impact several disease-causing genes via acquired in vivo mosaicism or acquired in vivo mutation, as is reported in Rothmund-Thomson syndrome (38).

Medical researchers are encouraged to report cases of other genetic diseases or chromosomal abnormalities accompanying WS, as doing so may help to identify which diseases are associated with WS.

References


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Appearance of high-molecular weight sialoglycoproteins recognized by *Maackia amurensis* leukoagglutinin in gastric cancer tissues: A case report using 2-DE-lectin binding analysis

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**Summary** Aberrant expression of sialoglycoconjugates has been thought to play an important role in cancer progression. Our previous lectin-histochemical study showed that overexpression of sialoglycoconjugates recognized by α₂,3-sialic acid-specific *Maackia amurensis* leukoagglutinin (MAL) was significantly related to the malignancy of gastric cancer. The present study analyzed the sialoglycoproteins in gastric cancer tissues by 2-dimensional electrophoresis (2-DE) in combination with lectin-binding analysis using MAL. Various MAL-positive sialoglycoproteins were detected in cancer tissues but not in non-cancer tissues. The sialoglycoproteins have a high molecular weight of near 200 kDa and over 200 kDa with different pI values for the two. This suggests that the MAL-positive sialoglycoproteins detected in gastric cancer tissues have high molecular weights and may contain different numbers of α₂,3-linked sialic acid residues in the carbohydrate moiety.

**Keywords:** Sialoglycoproteins, *Maackia amurensis* leukoagglutinin, Lectin, 2-Dimensional electrophoresis, Gastric cancer

1. Introduction

Sialoglycoconjugates, which bear sialic acid residues in their carbohydrate moieties, are essential in various biological events within organisms (1). Overexpression of sialoglycoconjugates and structural alteration of the carbohydrate moieties have frequently been detected in various cancer tissues and may be associated with tumor metastasis and progression (2-5). In this regard, detection of sialoglycoconjugates in tumor tissues with sialic acid-binding lectins would be helpful in evaluating the metastatic potential of those tumors and predicting patient prognosis. *Maackia amurensis* leukoagglutinin (MAL) recognizes α₂,3-linked sialic acid residues (6) and has been effectively used for biochemical and histochemical analyses of sialoglycoconjugates (7-9). A previous lectin-histochemical study on gastric cancer tissues by the current authors has suggested that MAL-positive sialoglycoconjugates were detected in cancer tissues but not in non-cancer tissues and that overexpression of the MAL-positive sialoglycoconjugates was related to worse prognosis for patients (10). However, the nature of MAL-positive sialoglycoconjugates in gastric cancer tissues has yet to be clarified. The present study characterized MAL-positive sialoglycoconjugates in gastric cancer tissues by means of 2-dimensional electrophoresis (2-DE) in combination with lectin binding analysis using MAL.

2. Materials and Methods

2.1. Tissues

Fresh gastric cancer and the corresponding non-cancer tissue samples (548 and 258 mg wet weight, respectively)
were collected from a patient who underwent surgical resection at the Department of Surgery, the University of Tokyo, Japan. Tissue samples were stored in -80°C until use for protein extraction or fixed with formalin followed by embedding in paraffin for lectin-histochemistry.

2.2. Lectin-histochemistry

Five-μm-thick sections were cut from formalin-fixed paraffin-embedded tissue blocks and subjected to lectin-histochemistry using 4 μg/mL of biotinylated MAL (Seikagaku Co., Tokyo, Japan) as described elsewhere (10). Detection was performed by a biotin-streptavidin-peroxidase complex method using 3,3’-diaminobenzidine as a chromogen and hematoxylin as a counterstain.

2.3. Protein extraction

Proteins were sequentially extracted from the tissue samples into 3 fractions based on the different solubility using a ReadyPrep sequential extraction kit (Bio-Rad Laboratories, Richmond, CA, USA). Briefly, the tissue sample was homogenized and sonicated with Reagent 1 (40 mM Tris base) followed by centrifugation at 5,000 rpm for 10 min at 4°C. The supernatant was obtained as a hydrophilic protein fraction. Next, the pellet was homogenized and sonicated with Reagent 2 (8 M urea, 4% (w/v) CHAPS, 0.2% SB3-10, 40 mM Tris) followed by centrifugation at 5,000 rpm for 10 min at 4°C. The supernatant was obtained as a slightly hydrophobic protein fraction. Finally, the pellet was then homogenized and sonicated with Reagent 3 (5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.2% SB3-10, 40 mM Tris) followed by centrifugation at 5,000 rpm for 10 min at 4°C. The supernatant was obtained as a highly hydrophobic protein fraction. The protein in each fraction was quantified with a Protein assay kit (Bio-Rad Laboratories) in accordance with Bradford’s method (11).

2.4. Immobilized pH gradient-2-DE

Individual fractions of protein samples were applied to an immobilized pH gradient (IPG)-2-DE. Briefly, 25 μg of sample protein were subjected to first-dimension isoelectric focusing (IEF) using a PROTEAN IEF system (Bio-Rad Laboratories) and a linear IPG strip (7 cm length, pH 3-10, Bio-Rad Laboratories). After electrofocusing, the gel strip was equilibrated with equilibration buffer I (0.375 M Tris-HCl, pH 8.8, containing 6 M urea, 20% (w/v) glycerol, and 2% (w/v) dithiothreitol) for 20 min and then with equilibration buffer II (0.375 M Tris-HCl, pH 8.8, containing 6 M urea, 20% glycerol, 2% SDS, 2.5% (w/v) iodoacetamide) for 10 min. The equilibrated gel strip was placed on a 7.5-15% gradient polyacrylamide gel and then second-dimension SDS-PAGE was carried out at 200 V for 40 min. To detect proteins, the gel was stained with SYPRO Ruby staining solution (Bio-Rad Laboratories).

2.5. Western blotting and lectin binding assay

After IPG-2-DE, proteins in the gel were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories) by using HorizBlot AE-6677 (ATTO, Tokyo, Japan). The membrane was blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 3 h at room temperature and then incubated with 5 μg/mL of biotinylated MAL in 1% BSA-TBS for 1 h at room temperature followed by incubation with streptavidin-conjugated horseradish peroxidase solution (Nichirei, Tokyo, Japan) for 1 h at room temperature. Protein spots were detected by an enhanced chemiluminescence (ECL) method (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

3. Results and Discussion

First, expression of MAL-positive glycoconjugates in gastric cancer tissues was examined histochemically. As shown in Figure 1, MAL-positive staining was observed only in cancer tissues (left side) but not in non-cancer tissues (right side), which is consistent with a previous report (10).

Next, proteins in the sample tissues were extracted and fractionized as described in Methods and then subjected to 2-DE. Lectin binding analysis using MAL was performed after the proteins were transferred from 2-DE gels to PVDF membranes. As shown in Figure 2A, the hydrophilic protein fraction of gastric cancer tissue yielded two series of MAL-positive spots that were detected at positions with a molecular weight of over 200 kDa and pI range of 4-6 and positions with

Figure 1. Typical example of lectin-histochemical staining using MAL. The stain was only detected in the cancer region (left) but not in the non-cancer region (right). Original magnification, ×200.
a molecular weight of near 200 kDa and pI range of 3.5-5.5. In contrast, distinct MAL-positive spots were not observed in the non-cancer tissue sample (Figure 2B). Two series of MAL-positive spots were also detected in the slightly hydrophobic protein fraction of cancer tissue (Figure 2C). The molecular weight profile of these proteins was similar to that detected in the hydrophilic fraction of cancer tissue, although the pI range of spots with a high intensity appeared to be close to the neutral point (Figure 2C). In contrast, MAL-positive spots were not observed in these areas in the slightly hydrophobic protein fraction from the non-cancer tissue sample (Figure 2D). A MAL-positive protein at a position with ~90 kDa and pI 5.5 detected in this fraction is thought to be derived from contaminated blood components such as leukocytes (12). In highly hydrophobic fractions, MAL-positive spots were not detected in both cancer and non-cancer tissues (data not shown).

A previous lectin-histochemical study with gastric cancer tissues by the current authors indicated that MAL-positive glycoconjugates exclusively appear in cancer tissues but not in non-cancer tissues, which was confirmed in the present study (Figure 1), and that the aberrant increase in expression is significantly correlated to invasion and metastasis of cancer cells and a worse prognosis for patients (10). Thus, analyzing the property of cancer-specific MAL-positive sialoglycoconjugates is crucial to clarifying the mechanism of cancer progression. A preliminary study by the current authors suggested that MAL-positive sialoglycoconjugates are contained in the protein fraction but not in the glycolipid fraction (data not published). Thus, the present study analyzed the MAL-positive sialoglycoproteins by means of 2-DE and lectin binding analysis.

As described above, two series of cancer-specific MAL-positive sialoglycoproteins with high molecular weights near 200 kDa and over 200 kDa, respectively, were detected in both hydrophilic and slightly hydrophobic fractions. The sialoglycoproteins in each series have similar molecular weights but have different pI values. Therefore, one possibility is that each series of sialoglycoproteins may have a same polypeptide backbone but possess different numbers of α2,3-linked sialic acid residues in its carbohydrate moiety. Although the detailed nature of these glycoproteins has not been determined, the high molecular weight of the MAL-positive sialoglycoproteins detected in gastric cancer tissues suggests that these sialoglycoproteins may be a type of mucins that are known to play a major role in cancer progression (13). In addition, the relationship between the expression of these sialoglycoproteins and cancer progression needs to be further investigated.
between clinical characteristics and 2-DE profile of MAL-positive sialoglycoproteins remains unclear. Proteomic analysis with 2-DE is a technique that has often been used to identify specific proteins (14,15) and has been developed to identify glycoproteins with a specific carbohydrate structure by using lectins (16,17). The current work shows the 2-DE profile of MAL-positive sialoglycoproteins obtained from one patient. Further studies with multiple specimens must be performed to clarify the clinical significance of MAL-positive sialoglycoproteins in gastric cancer tissues and determine their nature.

Acknowledgements

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Social work in international health and medical assistance

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Summary  Welfare issues such as the poor, children, women, and the handicapped are dealt with in the field of development assistance. Few studies, however, have discussed development assistance from a social work point of view. This study analyzes the social work aspects of development assistance through a review of 60 health projects completed by the Japan International Cooperation Agency between 2000 and 2006. Although the term "social work" is ambiguous, several projects with diverse themes included what could be called social work. Projects conducted three types of activities: that for a target population of social works; that for the general population, which included its target population; and that not for a specific target population. Project interventions included both micro-level interventions and system development. There are several possible reasons why only a few projects included social work: 1) social work has a lower priority in development assistance than other areas such as health do, and 2) there are few relevant specialists who can handle a wide range of social work interventions.

Donor agencies are gradually focusing more on social work aspects in their projects. Since social work will likely become a greater necessity in the field of development assistance for developing countries in the near future, donor nations and agencies will need to be prepared more adequately to respond to social work needs.

Keywords: Social work, Social welfare, Development assistance, Developing countries, Health

1. Introduction

In recent years, social welfare has become an aspect of development assistance studies. At the Lyon Summit focusing on social welfare, Prime Minister Hashimoto of Japan announced the Initiative for a Caring World (1). In 1996, the East Asian Ministerial Meeting on Caring Societies was held in Okinawa (2,3). Following these initiatives, Japan launched the Community Empowerment Program as a part of its social welfare aid (4). This trend has also appeared in other aid agencies such as the World Bank and Asian Development Bank (5). Since 1996, the World Bank has increased investment in the social welfare sector (5).

According to the National Association of Social Workers in the United States (6), social welfare is defined as "a nation's system of programs, benefits, and services that help people meet those social, economic, educational, and health needs that are fundamental to the maintenance of society." This social welfare framework encompasses the poor (7-11), child care (7,12), child abuse (13), child trafficking (14), street children (3,15), widows (7), unsafe abortion (16), victims of sexual violence (17) and domestic violence (18), the elderly (5,7,19-21), the handicapped (7,22), the homeless, people living with HIV/AIDS and their families (23,24), disaster survivors (25), immigrants, refugees (26), minorities, alcoholics (27), and drug addicts. There are welfare laws (7,28) dealing with these issues, and public welfare programs exist to provide a wide range of services (22).

Social work, which is defined as "the professional activities of helping individuals, groups, or communities to enhance or restore their capacity for social functioning and to create societal conditions favorable to their goals" (1), can play an important role...
in the social welfare system for such people (23).

In the field of development assistance, however, the issues affecting such people are considered to fall under social work but instead fall under health, insurance, labor, disabilities, education, gender, district health system development, refugee-related issues, etc. (5,29). As in developed countries, furthermore, social work services do not cover all of the groups previously listed.

Here, the population which social work targets will be referred to as the "target population." All 60 JICA technical cooperation projects on health that were conducted by the Japan International Cooperation Agency (JICA) and completed between 2000 and 2006 were reviewed (see Table 1) (30-89) in order to analyze the social work aspects of these projects in the context of development assistance.

2. Scope of social work

2.1. Historical changes in the role of social work

In the past, development assistance has not been discussed from the point of view of social work or social welfare. Perhaps one reason for this is because the terms "social welfare" (5) and "social work" are ambiguous. For instance, distinguishing social welfare activities from other regular human services is sometimes difficult. That is, regular human services such as library services, consumer protection, and firefighting are not recognized as social welfare services, and the term "human services" is broader than what is encompassed by social welfare programs.

In this ambiguous framework of social welfare, the meaning of the term "social work" is also unclear. Another reason for this ambiguity is that the issues which social work deals with and its interventions have changed gradually over time in response to historical changes in social needs. In the early 1800s, the target population was the poor, and in the United States and other developed countries interventions focused more on physical needs, such as food and shelter. Later, the target population broadened to include the unemployed, the sick, the physically and mentally handicapped, and orphans. This led to interventions in the form of social casework and family counseling. Later still, community organization and social planning approaches were introduced to deal with social problems (6). Consequently, the target population has changed over time since "there is a tendency to use the term 'human services' for what in the past has been called 'social welfare'" (6,22). Today, therefore, the target population differs from country to country (28).

2.2. Population approach to social work

Should activities such as primary health care and mobile clinics for remote areas in developing countries be considered social work or regular human services? JICA classifies primary health care as one activity to reduce regional disparities and poverty in some of its projects (in Zambia, Nicaragua, China, etc.). Ullin has also noted that primary health care requires a greater team approach, integrating nutrition, agriculture, social work, education, and other fields (90). In reality, many people in remote areas of developing countries live in poor and precarious living conditions and have limited access to human services. This means that there are needs to which social work services should respond in developing countries, although these may be covered by regular human services in developed countries. In this paper, primary health care and health promotion activities, including community participation approaches, are classified as activities for the general population, which includes the target population, whether they include social work or not. This is since the Ottawa Charter (91) states that "health promotion is not just the responsibility of the health sector but goes beyond healthy lifestyles to well-being."

3. Classification of "social work"

3.1. Classification of the "target population"

A target population can be classified into three types: "individuals," "families," and "population segments."

1) An individual target population includes persons who are not necessarily related but who are suffering from a similar problem, such as a disability, homelessness, or domestic violence. Examples of work targeting an individual population are domestic violence counseling (Honduras) and mass health examinations for radiation victims (Kazakhstan).

2) A family target population includes persons suffering from difficulties as a family, such as broken or bereaved families and the poor. Examples of work targeting a family target population are user fee exemptions for the poor (Cambodia), X-ray diagnostic service for the poor (Dominican Republic), and support for AIDS widows (Thailand).

3) A population segment target population includes certain population segments (race/ethnicity, sex, geography, etc.) suffering from difficulties such as discrimination. An example of work targeting a population segment is income generation for women (Jordan).

Although, this study covered all three types of target populations, the most common interventions were for a population segment (two projects targeted an individual population, five targeted families, and 18 targeted a population segment).
3.2. Classification of project activities and projects

1) Project activity classification by target population: There are three types of project activities from a social work point of view (Figure 1).

Type 1: Those that do not target a target population,
Type 2: Those for the general population, which includes a target population,
Type 3: Those that target a target population.

Type 1 activities included upgrading a clinical laboratory, Type 2 activities included primary health care activities for people living in rural areas, including the poor, and Type 3 activities included domestic violence counseling training, establishing counseling systems, and setting up a user free exemption system for the poor.

2) Project Classification: Based on the types of project activities, the projects themselves can be classified into the following three categories.

Category 1: Projects with only Type 1 activities,
Category 2: Projects with Type 2 activities and possibly including Type 1 activities,
Category 3: Projects with Type 3 activities and possibly including activities of other Types.

Table 1 shows the classification of each project. Out of 60 projects, there were 35 Category 1 projects (59%), 17 Category 2 projects (28%), and eight Category 3 projects (13%) indicating that only a small number of projects involved social work targeting a specific population.

In addition, the study results show that Category 3 project themes are diverse, covering areas from improving maternal and child health and enhancing district health systems to controlling infectious diseases, indicating that many projects can be considered to include social work components regardless of the project theme.

3.3. Classification of interventions

Here, project activities are classified as either micro-level intervention, such as case work, case management, group work, group therapy, and family therapy, and system development or policy-making. Social work interventions in the form of both micro-level interventions and system development or policy-making were observed in health-related development assistance projects. The activities in Category 3 included activities for case work services for individual clients, such as technical training for counseling in Honduras and Jordan and the provision of X-ray diagnostic services in the Dominican Republic, and activities for system development, such as creating a user free exemption system for the poor in Cambodia. The Honduras project covered both case work services and system development for counseling services.

Different levels of assistance activities are therefore necessary for developing countries since no special national social work or social welfare system usually exists nor are there official social workers in these countries (5). Therefore, the system development approach appears useful. In this context, UNICEF has recently conducted special seminars in Myanmar to train social workers and to help improve social work proficiency and establish a social work system (92). The current findings also show that there are several project activities relating to development of the social welfare system rather than micro-level interventions in Category 3 projects. This indicates that policy-making and system development related to social work are likely to become more important in developing countries in the near future.

4. Importance of social work

Japan has conducted several projects to establish social welfare systems for the elderly and for street children and to develop national insurance systems in developing countries, although, as with other donor nations, it has supported only a handful of aid projects focusing on social work (5). There may be several possible reasons for this. First, social work has a lower priority in development assistance than do other areas such as health. In other words, disease mortality and morbidity are greater concerns in developing countries than quality of life, which social work focuses on. That said, several facts are clear: the problem of poverty is related to health (93-95), and the issue of the elderly will become more pressing in developing countries in the near future (20,96). Consequently, social work as part of international health assistance will receive greater attention.

Second, there are very few relevant specialists. Japan, for instance, has dispatched several policy-making advisors and Japan Overseas Cooperation
Table 1. Project list

<table>
<thead>
<tr>
<th>Year of end</th>
<th>Country name</th>
<th>Project name</th>
<th>Project theme</th>
<th>Social work related interventions</th>
<th>Category</th>
<th>Level of target group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Costa Rica</td>
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<td>Clinical</td>
<td>Mass screening</td>
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<td>Population segment</td>
</tr>
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<td>Training of primary health workers</td>
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<td>Population segment</td>
</tr>
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<td>3</td>
<td>Population segment</td>
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<tr>
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<td>Vietnam</td>
<td>Reproductive health project in Nghe an province</td>
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<td>Mobile team, community participation</td>
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<td>Population segment</td>
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<td>PHC, community participation to protect women</td>
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(to be continued)
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<td>Nicaragua</td>
<td>The project for strengthening of local system of integral health care (SILAIS) of Granada</td>
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<td>Community participation, health promotion</td>
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<td>Dominican Republic</td>
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<td>Madagascar</td>
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<td>Family</td>
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<td>Cambodia</td>
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<td>Family</td>
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<td>The Republic of Honduras</td>
<td>The reproductive health project in the Health Region Seven</td>
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<td>PHC for the poor</td>
<td>2</td>
<td>Population segment</td>
</tr>
<tr>
<td>53 2005</td>
<td>Thailand</td>
<td>The project for the Asian center of international parasitic control</td>
<td>Infectious diseases</td>
<td>None</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>54 2005</td>
<td>Myanmar</td>
<td>The project for primary health care for mothers and children in Myanmar</td>
<td>MCH/Repro</td>
<td>Nutrition and food program</td>
<td>2</td>
<td>Population segment</td>
</tr>
<tr>
<td>55 2005</td>
<td>Vietnam</td>
<td>Bach Mai Hospital project for functional enhancement</td>
<td>Hospital</td>
<td>None</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>56 2005</td>
<td>China</td>
<td>The expanded program on immunization strengthening project</td>
<td>Infectious diseases</td>
<td>None</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>57 2006</td>
<td>Zambia</td>
<td>Cross border initiative project</td>
<td>Infectious diseases</td>
<td>Peer educator, drop-in center</td>
<td>3</td>
<td>Population segment</td>
</tr>
<tr>
<td>58 2006</td>
<td>Zambia</td>
<td>HIV/AIDS and tuberculosis control project</td>
<td>Infectious diseases</td>
<td>None</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>59 2006</td>
<td>Thailand</td>
<td>The project for strengthening of national institute of health capabilities for research and development on AIDS and emerging infectious diseases</td>
<td>Infectious diseases</td>
<td>Support for AIDS widows</td>
<td>3</td>
<td>Family</td>
</tr>
<tr>
<td>60 2006</td>
<td>Bolivia</td>
<td>The project for strengthening Regional health network for Santa Cruz Prefecture in the Republic of Bolivia</td>
<td>District health</td>
<td>Health promotion for remote areas</td>
<td>2</td>
<td>Population segment</td>
</tr>
</tbody>
</table>
Volunteers in the field of the elderly and street children (5), although few specialists on social work have been dispatched as members of missions to evaluate health projects. The current findings suggest that various aspects of social work, from case work and community organization to system development and policy-making, are required in developing countries. Experienced generalists in social work can assist with those aspects (6). In Japan, few experts in social work have received such specialized training (5). While donor agencies may recognize the necessity of social work in projects, such specialized training and education still needs to be fostered in Japan.

5. Conclusion

This study shows that social work is already being implemented in various ways, although the amount of this work appears inadequate. Since the importance of social work in development assistance in developing countries is likely to increase in the near future, donor nations and agencies will have to prepare themselves more adequately to respond to social work needs.

Acknowledgement

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Quick detection of herpes viruses from skin vesicles and exudates without nucleic acid extraction using multiplex PCR

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Summary
Distinguishing herpes virus infection from other skin diseases is sometimes difficult. This study aims to detect herpes virus DNA by multiplex real-time PCR without nucleic acid extraction in a short period of time. Specimens of cutaneous vesicles and swabs were obtained from 23 patients suspected of having herpes virus infection. These specimens were stored at -80°C after dissolving them in sterilized water. DNA extraction was not performed. Specific real-time PCR primers for herpes simplex virus (HSV) 1 and 2 and varicella-zoster virus (VZV) were designed. These primers were used to perform real-time PCR with the frozen solution as template. Results clearly revealed a type-specific dissociation curve. Agarose gel electrophoresis was also performed and produced a single band of the expected size. In addition to using multiplex PCR, other steps were used to reduce the time even further. Each experiment took only 2 h to complete; the type of Herpes virus was successfully detected by multiplex real-time PCR without nucleic acid extraction in a short period of time. In conclusion, omission of the nucleic acid extraction step prior to real-time PCR does not negatively affect downstream reactions. Using multiplex PCR may allow more rapid qualitative analysis of HSV1, 2 and VZV.

Keywords: Herpes virus, Real-time PCR, Multiplex PCR

1. Introduction
Occasionally, skin lesions may present problems in terms of their diagnosis. Distinguishing herpes simplex virus infection from herpes zoster infection or other skin diseases is sometimes difficult. Rapid and accurate detection and typing of herpes simplex virus type 1 (HSV-1), type-2 (HSV-2) or varicella-zoster virus (VZV) is crucial to clinical diagnosis and therapy as early as possible. In Japan, the tests most commonly used for the diagnosis of herpes virus infections of the skin are the Tzanck test and immunofluorescence of the serum. However, the Tzanck test does not distinguish between herpes simplex and varicella-zoster virus. The immunofluorescence method only has a sensitivity of 32% (1). Herpes viruses are DNA viruses, and therefore no extra procedures are required to obtain DNA, providing a significant reduction in the cost and time involved in the experimental procedure. The possibility of contamination of the sample during the DNA extraction procedure is also eliminated. Several recent reports have indicated that extraction and purification of nucleic acid is not always necessary to perform real-time PCR (2,3). To the extent known, there are no reports in the field of dermatology that have discussed omission of DNA extraction, but there are reports in other fields (4,5). The liquid from cutaneous vesicles and exudates from patients with a possible diagnosis of HSV or VZV infection was examined. Uniplex PCR was initially used; multiplex PCR was later performed on the same specimens using the same primers.

2. Materials and Methods
2.1. Samples
In the period from December 2006 to September 2007, clinical specimens (n = 25) suspected for herpes viruses...
infection were collected from outpatients and inpatients at Kumamoto University Hospital (Table 1). Specimens were collected after rupturing the skin vesicles and swabbing erosions or ulcers from various anatomical sites. Vesicle skin samples were dipped into 100 μL of sterilized water and swabs were washed in 400 μL sterilized distilled water and discarded. The solution alone was used as a template, and specimens were stored at -80°C.

2.2. Primer and settings

The reaction mixture contained 0.5 μL of specimen, 11 μL of deionized water, 0.5 μL each of the forward and reverse primer, and 12.5 μL of Ex Taq DNA polymerase (SYBR® Premix Ex Taq™, TaKaRa, Shiga, Japan). The primer sequences used are shown in Table 2. All reactions were performed in a Thermal Cycler Dice® Real Time System (TaKaRa). The thermal cycler was pre-heated to 95°C for 10 sec followed by 40 cycles of annealing at 95°C, extension for 30 sec at 60°C, and denaturation for 5 sec at 95°C. Dissociation was performed at the end of this reaction.

2.3. Agarose gel electrophoresis

PCR amplification was examined with agarose gel electrophoresis. Five-μL aliquots of the amplification products from each primer were run on 2% Agarose gel (Ultra PURE Agarose, Life Technologies, Gaithersburg, USA).

2.4. Uniplex PCR

Initially, all specimens were examined for HSV1, HSV2 and VZV infection by uniplex real-time PCR. Five or six specimens were examined at a time, with each PCR run lasting 2.5 h. For all 25 specimens, PCR was performed 6 times.

2.5. Multiplex PCR

A master mix containing forward and reverse primers for all viruses, deionized water, and polymerase was made. Aliquots of 0.5 μL were taken from all 25 specimens, 22 μL of master mix was added, and multiplex PCR was performed with a Thermal Cycler.
3. Results

Table 1 shows the results of real-time PCR for 25 specimens obtained from 23 patients. Figure 1 shows a typical clinical profile of HSV 1, HSV 2, and VZV patients, respectively. Figure 2 shows the respective amplification plots and dissociation curves for patients no. 15, 16, and 19 using uniplex PCR. Nineteen of the 25 specimens (76%) were positive for one of the three target viruses; HSV 1 \((n = 4)\), HSV 2 \((n = 3)\), VZV \((n = 12)\), respectively. Their dissociation curves had an exact peak temperature specific to each virus DNA: 84.25°C for HSV1, 87.75°C for HSV2, and 79.75°C for VZV. The experiments took about 2.5 h to complete using uniplex PCR, and experiments were performed 6 times to obtain results for all 25 specimens.

All of the 25 specimens were retrospectively analyzed at a time using multiplex PCR. Nineteen of the 25 specimens (76%) were positive for one of the three target viruses (HSV 1 \((n = 4)\), HSV 2 \((n = 3)\), and VZV \((n = 12)\)). Figure 3 shows the respective amplification plots and dissociation curves for patients no. 15, 16, and 19.

Figure 1. Clinical presentation of patients. (a) Patient no.16. A bullous lesion was present on her upper lip (HSV1). (b) Patient no.15 (HSV2). (c) Patient no.19. A bullous lesion was present in his left femoral area (VZV).

Figure 2. Amplification plots and dissociation curves for three patients by uniplex PCR. (a, b) Patient no.15 sample with HSV2-specific primers. Peak temperature of 88.75°C on the dissociation curve. (c, d) Patient no.16 sample with HSV1-specific primers. Peak temperature of 84.25°C. (e, f) Patient no.19 sample with VZV-specific primer. Peak temperature of 79.75°C.
using multiplex PCR. Multiplex PCR results for all of the positive specimens were exactly the same as those obtained with uniplex PCR in terms of the dissociation curve and temperature (Table 1, Figure 4). Analyzing all 25 specimens took only 2 h. Multiplex PCR reduced the experimental time 30 min although the number of specimens increased.

Frozen solutions of the sample were able to withstand multiple freeze-thaw cycles; this was confirmed by results indicating the same dissociation curves, even for specimens that were stored for up to 11 months.

Agarose gel electrophoresis was performed to check the bands of PCR amplification. Five microliters of the amplification products were run in 2% agarose gel, and the results are shown in Figure 5. The difference in band sizes was reflected by the migration distance, which also confirmed the dissociation curve results.

Figure 3. Amplification plots and dissociation curves for three patients by multiplex PCR. (a, b) Patient no.15 (HSV2) sample with master mix. Peak temperature of 88.75°C on the dissociation curve. (c, d) Patient no.16 (HSV1) sample. Peak temperature of 84.25°C. (e, f) Patient no.19 (VZV) sample. Peak temperature of 79.75°C.

Figure 4. Dissociation curves of all positive specimens (n = 19). Each curve had only one peak temperature, and these temperatures were exactly the same as those obtained with uniplex PCR.
4. Discussion

Nucleic acid extraction is expensive, time-consuming, and may result in specimen contamination prior to analysis. To counter such problems, this study has described a reliable and speedy way of confirming the diagnosis of herpes viruses without nucleic acid extraction.

Real-time PCR is a speedy and sensitive method for the detection and genotyping of infectious diseases such as herpes viruses. Earlier methods include isolation of the virus in cell culture and detection of the virus followed by immunofluorescence microscopy, but this method was laborious. In the current study, clinical specimens of skin vesicles and swabs from patients suspected of being infected by herpes viruses (HSV type 1/2, VZV) were analyzed by real-time PCR without nucleic acid extraction or purification. Mark et al. investigated PCR performance with or without nucleic acid isolation from specimens (2). They found only one peak of the dissociation curve, which means that omission of DNA extraction did not negatively affect data analysis. Omission of the DNA extraction step provides a significant reduction in time and cost.

Specific primers for each virus amplified each specific DNA arrangement and were distinguishable by dissociation temperature. A stock cocktail containing the primers of both (HSV1/2, VZV), polymerase, and de-ionized water was prepared to identify ways to further reduce the experimental time, cost (of tubes), and possibility of contamination. As shown in Figure 4, the same dissociation curve for each positive specimen was obtained by using multiplex PCR. Recently, the use of multiplex PCR for the diagnosis of herpes has become more and more common (4-8). Several reports compared uniplex and multiplex PCR (4, 6). Multiplex PCR was found to be more rapid, specific, and sensitive than uniplex PCR. Multiplex PCR assay also offers increased sensitivity, typing, and improved turnaround time compared to traditional viral culture and immunofluorescence techniques (8).

5. Conclusions

The current results indicate that omission of the nucleic acid extraction step prior to real-time PCR does not negatively affect downstream reactions. Multiplex PCR is a rapid, sensitive, time-saving, and cost-effective assay when many specimens are being examined. A sample/water solution was stored in a -80°C freezer for several months without deterioration in quality, even after several freeze-thaw cycles.

On the basis of these results, work to devise methods to further reduce experimental time will continue.

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