Table 2 Up and down regulations of erythropoietin-producing hepatoma receptors in human cancers [+]

	Up-regulation		Down-regulation		
	Overexpression	Amplification	Promoter methylation	Loss of heterozygosity	Others or unknown
EPHA1	Stomach		Colon	Colon	Colon
EPHA2	Stomach, colon, esophagus			Colon	(Melanoma)
EPHA3			(Lung)		
EPHA4	Colon				
EPHA5					
EPHA6					
EPHA7	Stomach, colon		Stomach, colon		
EPHA8				Stomach, colon	
EPHB1					
EPHB2	Stomach, colon	Stomach	Colon	Colon	(Prostate)
EPHB3		Colon			
EPHB4		Colon	Colon	Colon	
EPHB5					
EPHB6	(Neuroblastoma)				

No published data in the blanks. Parenthesis indicate non-GI tract cancers. EPH: Erythropoietin-producing hepatoma.

Table 3 Receptors and ligands corresponding

	EFNA1	EFNA2	EFNA3	EFNA4	EFNA5	EFNB1	EFNB2	EFNB3
EPHA1	x							
EPHA2	x	x	x	x	x			
EPHA3	x	x	x	x	x			
EPHA4	x	x	x	x	x		x	x
EPHA5	x	x	x	x	x			
EPHA6		x						
EPHA7		x			x			
EPHA8	x	x	x	x	x			
EPHB1						x	x	x
EPHB2						x	x	x
EPHB3						x	x	x
EPHB4						x	x	x
EPHB5								
EPHB6						x	x	x

x indicates a known interaction. Each binding constant is shown in the reference Bowden *et al*[27]. EPH: Erythropoietin-producing hepatoma.

46 Japanese patients with colorectal cancer reported by Shao *et al*[22]. Cell signaling studies using a culture system disclosed a role of *EPHA3* in the formation of a cell's shape[23]. Thus, changes in *EPHA3* are likely to produce particular morphological and biological characteristics in the tumor cells carrying these changes, although no correlation between the *EPHA3* status and the clinico-pathological features of gastrointestinal cancers has yet been described. Although the clinical relevance is unknown, there is a report investigating the LINE-1 methylation pattern in the introns of *EPHA3* in tumor cells[24].

EPHA4

The over-expression of *EPHA4* has been reported in gastric and colorectal cancers[25,26]. In both cancers, the over-expression of *EPHA4* is an ominous sign with a shorter survival period and frequent liver metastasis respectively. *EPHA4* is the only type A receptor that binds a B family ligand, EPHRIN(EFN)B2, in addition to a type A ligand, EPHRIN(EFN)A2 (Table 3). A structural study has been conducted to reveal the stereoscopic interactions between several members of EPH receptors and EPHRIN(EFN)s[27]. The potential significance of *EPHA4* over-expression in clinical oncology and the possibility of its use as a therapeutic target remain unknown.

EPHA5

There is no information regarding alterations in *EPHA5* in human gastrointestinal cancers. *EPHA5* is not expressed in the intestine at any age, as reported by Islam *et al*[28].

EPHA6

Research on *EPHA6* in the gastrointestinal tract is sparse. *EPHA6* is commonly expressed in the testis and brain[29].

EPHA7

Since the first description of the down-regulation of *EPHA7* in colorectal cancer[30], several papers have assessed the expression of *EPHA7* in human gastrointesti-

nal cancers[31], human lung cancer[32] and prostate cancer[33]. The biological basis for these clinicopathological observations and their significance in oncology remain to be investigated. The promoter methylation of *EPHA7* was the first example of down-regulation by methylation in *EPH* receptors but a subsequent survey of other *EPH* receptors, including *EPHB* receptors in colon cancer, produced negative results[34]. Another topic concerning *EPHA7* is its secretory form. The secretory form of *EPHA7* contains only the extracellular part of the molecule and does not anchor at the cell membrane. Its biological and clinical significance remain unknown. A secretory form of *EPHA7* is known to exist in malignant lymphoma[35] and lung cancer[32] but no study has been conducted on the presence of the secretory form of *EPHA7* in clinical gastrointestinal cancer.

Although the clinical significance is still unclear, Kim *et al*[36] reported a single nucleotide polymorphism (SNP) at the *EPHA7* locus, rs2278107; this SNP was related to the chemoresponsiveness to fluoropyrimidine-based adjuvant chemotherapy for colorectal cancer[36].

EPHA8

EPHA8 was screened for mutation in Japanese colorectal cancer but no mutations were found[22], similar to other *EPHA* receptors such as *EPHA3* and *EPHA7*. The *EPHA8* receptor induces the sustained up-regulation of MAP kinase; thus, it is supposed to play a role in tumor cell growth and proliferation[37]. *EPHA8* is expressed during the fetal period of intestinal morphogenesis[28] and missense mutations in stomach cancer and colon cancer are known (Table 1).

EPHB1

EPHB1 has been investigated in terms of signal transduction involved in the biological behavior of tumor cells[38,39], but little information is available on its status in human clinical cancer. An *EPHB1* mutation was recently identified in ovarian cancer and missense mutations have also been found in gastric cancer[40] (Table 1).

EPHB2

EPHB2 is the most extensively studied member of *EPH* receptors in the field of oncology. Kiyokawa *et al*[2] reported the overexpression of *EPHB2* in human gastric cancer and assigned it to the chromosomal locus at 1p36 which many investigators have assumed to be a tumor suppressor locus of human colon cancer because of the frequent loss of heterozygosity that has been documented[41]. Subsequently, Oba *et al*[42] demonstrated the loss of heterozygosity of the *EPHB2* locus in human colorectal cancer. Furthermore, Batlle *et al*[43] argued that *EPHB* receptor activity could suppress the progression of colorectal cancer and *EPHB2* is now viewed, at least in some contexts, as a tumor suppressor or a suppressor against tumor progression[26,44-48], although different aspects have also been discussed[49]. A group led by Hans Clevers put forward the comprehensive idea of *EPHB2-EPHRINB1* interplay at the bottom of human colon crypts[50,51]. They showed the clear territory of *EPHB2* and *EPHRINB1* in a human colorectal crypt, its important role in cell positioning and the ordered developmental migration of intestinal cells using *EphB2/EphB3* knockout mice[51]. This view is now prevalent[52] and they have further refined the concept of a stem cell unit in human gastrointestinal crypts[53,54]. Based on the mutually exclusive localization of *EPHB2* and *EPHRINB1*, Cortina suggested that tumor compartmentalization arising from the repulsive action of cells expressing *EPHB2* and *EPHRINB1* is a possible mechanistic basis for tumor suppression by the *EPHB2-EPHRINB1* system[55].

Then, what happened to the previous interpretation for the over-expression of *EPHB2* in human cancer[2,56,57]? Mao[58] reported *EPHB2* as a therapeutic antibody drug target for *EPHB2* over-expressing tumors. Mutation analyses in kinase genes have been very popular and somatic mutations of *EPHB2* have also been reported in many cancers[59,60], including GI tract cancers[61,62].

However, these mutations occur mostly in the microsatellite repeats of tumors with microsatellite instability or nonsense mutations causing RNA decay. No naturally occurring missense mutation that may positively or negatively influence the kinase activity of *EPHB2* has ever been reported. At this moment, we can only say that individual tumors may have an individual *EPHB2* status in an individual environment. The prevalence of methylation in the *EPHB2* promoter, on the other hand, is low compared with RASSF2 and O-6-methylguanine-DNA methyl transferase (MGMT) in early colorectal tumors[63].

There are reports investigating the possible contribution of germline *EPHB2* variants to rare polyposis syndrome[64,65]. The detailed mechanistic basis controlling the *EPHB2-EPHRIN* (*EFN*) *B1* system has also been investigated. Tanaka *et al*[66] reported that C-terminal *EFNB1* regulates matrix metalloproteinase secretion and that the phosphorylation of *EFNB1* regulates the dissemination of gastric cancer cells in an animal model[66]. He also showed the successful suppression of peritoneal dissemination in an animal model using an *EFNB1*-derived peptide[67]. The translational approaches using this method (use of EFNB1 peptide to suppress human cancer dissemination) have not yet been shown.

EPHB3

The localization and function of *EPHB3* partially overlaps with *EPHB2* in a Paneth cell compartment. *EPHB3* also has *EFNB1* as a ligand. Both are controlled by the beta-catenin/Tcf4 pathway[51].

Chiu reported that the over-expression of *EPHB3* enhanced cell-cell contact and suppressed tumor growth in HT-29 human colon cancer cells[68]. The defect in the positioning of Paneth cells is thought to arise from the disruption of the *EPHB2-EPHB3* system[69]. Clinicopathological information on *EPHB3* alone (not accompanied with *EPHB2*) in human gastrointestinal tract cancers remains limited. A clinical interpretation of the over-expression and/or amplification of *EPHB3* (Figure 1) in gastrointestinal cancer[70,71] awaits further investigations.

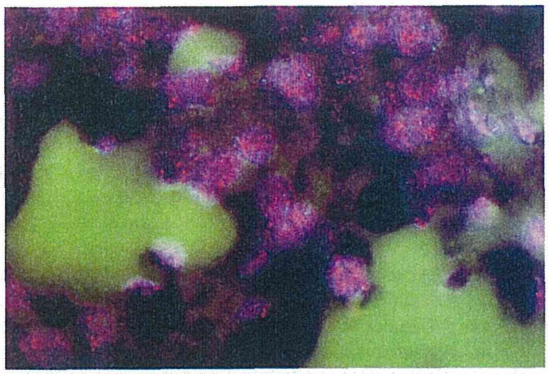

Figure 1 Fluorescence in situ hybridization of *EPHB3* (bacterial artificial chromosome RP11-328G15, red) in gastric cancer cells. Numerous red signals (more than 5) in a cell with two centromeres (green), indicating *EPHB3* amplification, are shown. The methodological details have been previously reported[69,70].

EPHB4

Kumar reported that *EPHB4* over-expression is more prevalent than *EPHB2* over-expression and the cyclic AMP-responsive element binding protein-binding protein (CBP) complex reciprocally regulates *EPHB2* and *EPHB4* (CBP complex suppresses *EPHB2* and induces *EPHB4* expression)[48]. *EPHB4* is thought to act in an *EPHB4-EPHB6* system[72] to regulate cancer cell invasiveness. The structure and dynamism on *EPHB4-EFNB2* was investigated[73,74] and the translational application of this basic knowledge awaits further investigation.

EPHB6

EPHB6 is the oldest *EPH* family member to attract enthusiastic interest from cancer researchers, especially neuroblastoma researchers. *EPHB6* is unique in that there is no kinase activity. It is one of the major genes involved in the clinico-biological behaviors of neuroblastomas[75-77]. Unlike other *EPHBs*, a suppressor role of *EPHB6* has been pointed out from an early stage of research[78-80], although its over-expression has been identified in leukemic cells[81]. A functional enigma of *EPHB6*, a kinase defective receptor affecting tumor invasiveness, has been gradually clarified in the fields of lung cancer research[82] but the role of *EPHB6* in carcinogenesis in the human digestive tract is not clear, although its alteration such as promoter methylation in lung adenocarcinoma, has been recently reported[83]. Recently, some missense variants have been reported in familial colorectal cancer[84]. Somatic changes in colorectal cancers according to ethnic stratification have revealed *EPHB6* to be one of the most frequently deleted genes in African Americans[85].

EPH RECEPTORS AS THERAPEUTIC TARGETS

Choi *et al*[86] reported the discovery of *EPHB2* receptor kinase inhibitors. They also performed crystallographic analyses of *EPHA3* and *EPHA7* in complex with their inhibitors and discussed the possibility of generating new inhibitors using a structure-based design[86]. This discovery and other structural studies[27,73,87] should pave the way for the development of drugs that specifically inhibit tumor cells over-expressing these receptors.

EPHA2 has been considered as a target for anti-angiogenesis therapy for a long time[8,88-92]. The EPHA2-Fc receptor was used to inhibit an *EFNA1-EPHA2* forward signal and to reduce neovascularization in rodent retina[91].

CONCLUSION

Although the *EPH* family is well known to be involved in the development of neural and vascular systems, their pivotal contributions to cancer biology, especially in clinical settings, remain to be elucidated. Enthusiasm regarding the use of *EPHs* as cancer therapy targets remains less than that of expectations for other groups of kinase receptors such as *EGFR*, *HER2*, *MET* and *RAF*[40,93]. The unique biological nature of *EPHs* such as bidirectional signaling and the presence of a secreted form, however, may provide a possible clue to manipulating the regulation of *EPH-EPHRIN* systems for human gastrointestinal cancer therapy. Gastrointestinal cancers have a special niche in Asian diseases in terms of their heterogeneity and uniqueness in etiology, both genetic and environmental[94]. An extensive search of EPH-EPHRIN systems in Asian gastrointestinal cancer patients will provide an important tool for the clinical management of Asian gastrointestinal cancer patients.

The real scale of the involvement of those genes in carcinogenesis in the human gastrointestinal tract still remains unclear and several research groups including Asians continue the search for molecular alterations of the EPH-EPHRIN system that may be relevant to detection and treatment of gastrointestinal cancers. The information stated here will be updated every year in future.

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Review Article

Fluorescence *in situ* hybridization analysis with a tissue microarray: 'FISH and chips' analysis of pathology archives

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Practicing pathologists expect major somatic genetic changes in cancers, because the morphological deviations in the cancers they diagnose are so great that the somatic genetic changes to direct these phenotypes of tumors are supposed to be correspondingly tremendous. Several lines of evidence, especially lines generated by high-throughput genomic sequencing and genome-wide analyses of cancer DNAs are verifying their preoccupations. This article reviews a comprehensive morphological approach to pathology archives that consists of fluorescence *in situ* hybridization with bacterial artificial chromosome (BAC) probes and screening with tissue microarrays to detect structural changes in chromosomes (copy number alterations and rearrangements) in specimens of human solid tumors. The potential of this approach in the attempt to provide individually tailored medical practice, especially in terms of cancer therapy, is discussed.

Key words: copy number alteration, fluorescence *in situ* hybridization (FISH), formalin-fixed paraffin- embedded (FFPE) tissue, pathology archives, tissue microarray (TMA)

INTRODUCTION

Extreme copy number alterations (aneuploidy) are the norm in human solid tumors.[1–3] Karyotyping solid tumors is so labourious[4] that only limited information on chromosomal abnormalities in human solid tumors *in situ* was available until recently. The latest methodologies that involve the use of human genome information, however, have provided us techniques that make it possible to identify any locus-specific chromosomal changes in a tumor. Several examples of applications of these state-of-the-art methodologies are essential diagnostic tools in diagnostic laboratories to, for example, identify translocation in certain solid tumors.[5–7]

New information is being obtained every day in genetic research on human solid tumors (especially carcinomas). The high-throughput, 'genome-wide' approach to genetic changes in human tumors has been widely adopted in every branch of medicine, and it is now known that there are extensive somatic changes, including multiple point mutations,[8,9] copy number alterations,[10,11] and further complex rearrangements[12] in every kind of tumor. Since most of these somatic changes have been identified in the analysis of the DNAs of advanced primary tumors and tumor cell lines, questions about when and where these genetic changes occur during cancer development in the human body remain to be answered by pathologists. Human pathology archives contain specimens of human tumors in various stages of development, from the incipient stage to the metastatic stage, and they are a treasure trove in the post-human-genome-sequencing era. The know-hows of two methods are important, especially for diagnostic pathologists: intensive application of bacterial artificial chromosome (BAC) clones as probes that have exact 'addresses' in the whole genome and construction of tissue microarrays (TMAs) which consist of hundreds of tissue specimens on a single slide. Using a combination of these two know-hows is a strategy that facilitates identification of changes at any genomic locus in several hundreds of tissue samples at once.

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Use of some of the specific BAC probes has already acquired a niche in routine examinations in diagnostic laboratories as a means of verifying a diagnosis, selecting subjects for particular molecularly targeted therapies, and for predicting recurrence.[13–20] Use of BAC probes by diagnostic pathologists, however, is still not widespread because of the difficulty of accessing and making the BAC probes for interests of their own. In this article we review the various facets of the latest advances in the application of BAC probes to diagnostic pathology and describe some of our own experiences with using many BAC probes to investigate pathology archives. We think that using numerous BAC probes will soon become a popular diagnostic practice, the same as the current use of monoclonal antibodies.

Actually, several ambitious pathology laboratories around the world that possess these methods in their arsenals, have started to propose an agenda of TMA-FISH ('Fish and chips') approaches to tumor DNA analysis.[21–30] The recent observation of repositioning of chromosomal loci during carcinogenesis has further encouraged the analysis of human tumor specimen in various clinicopathological settings.[31,32]

APPLICATIONS OF FISH TO DETECTION OF COPY NUMBER ALTERATIONS IN HUMAN TUMORS IN PATHOLOGY ARCHIVES

The development and modifications of the FISH procedure, especially for use in formalin-fixed-paraffin-embedded (FFPE) tissues have been extensively reviewed.[30] Equivalent hybridization efficiency of probes for the arrayed pieces of tissue after different fixation times and storage methods is necessary to correctly evaluate copy number amplification. In many studies, the FISH procedure has been performed as a means of validation, that is, to verify amplification data generated by other methodologies, such as by quantitative PCR, array-based comparative genomic hybridization (aCGH), and single nucleotide polymorphism (SNP) arrays,[33] and comparisons between methods and the interpretations of the results obtained by each method have sometimes been a matter of controversy.[34,35] FISH analysis, especially of FFPE tissues, is often technically demanding, and standardized quality control, which is very important in practical settings, has just begun. There are large inconsistencies between the prevalence of amplification of well-known and familiar genes that we consider clinically useful and that are routinely used in practice without rigorous quality control guidelines.[34,35] Thousands of BAC clones are commercially available, and, in theory, any of them can be used as FISH probes. The BAC clones or labeled probes can be ordered from at least two Japanese companies (Advanced GenoTechs Co., Tsukuba, Japan; GSP laboratory, Kawasaki, Japan). When we use these BAC clones for FISH procedures in paraffin-embedded tissue sections, several steps must be carefully performed including labeling and hybridizing them to DNA. The BAC clone must be confirmed to be the correct one, because assignments of BAC clones often change to reflect the daily process of refining the human genome database. The information on exact location of each BAC probe according to the most recent Build (Build 37 in March, 2010) of the human genome is necessary. Although the reason is usually unclear, some BAC clones hybridize with multiple sites (more than 4) in normal interphase cells, and logically they cannot be used to evaluate human tumors. Thus, commercial BAC probes must be tested to determine whether they are hybridized to the two corresponding sites (or two pairs of the signals on the sister chromatids) in the metaphase chromosome spread before they are applied to human tissues containing cancer cells (Fig. 1). Sequencing of part of the BAC probes is of some help in further confirming the correctness of the BAC probes.

In addition to the above-mentioned hurdles to obtaining the right BAC probes, there is another stumbling block to completion of a FISH procedure: the labeling step. Several labeling methods are available, and some are commercially available and packaged in the form of a kit. Sufficiently efficient labeling is sometimes achieved only in an heuristic manner.

The following limitations in interpretation must be considered when using a FISH procedure to enumerate chromosomes in paraffin-embedded tissue sections. The signals can be weak for many reasons. Clinical practice has been standardized only for the system for detection of HER2 amplification in breast cancer cases.[36] The merits of protease treatment, microwave treatment, heating, and other treatments such as using various detergents have been debated. Some 'pre-treatment' kits are commercially available, but retrieval efficiency usually depends on the condition of the specimen, and individual adjustments must be made each time in each laboratory. For example, the recommended pretreatment to augment signal strength in the two kits available, the Hercep test (Abbott, Tokyo, Japan) and the HISTRA (Jokoh, Tokyo, Japan) are different.[37] Based on our own experience, one technical tip for generating stable, sensitive signals in pathology archives that have been fixed by various methods and stored for a long period is appropriate, careful pretreatment with protease.

Since overlapping cells and cells whose nuclei are partially cut cause miscounting of the numbers of signals, cut-off values must be set based on preliminary evaluation of the signals in several non-tumorigenic tissues.[38,39] Several quality controls are necessary before applying the new probes to clinical uses the same as for the HER2 probe.

MERITS OF TMAs FOR SCREENING BY FISH

The preparation of FISH probes is a tedious task that includes several hurdles described in the previous section

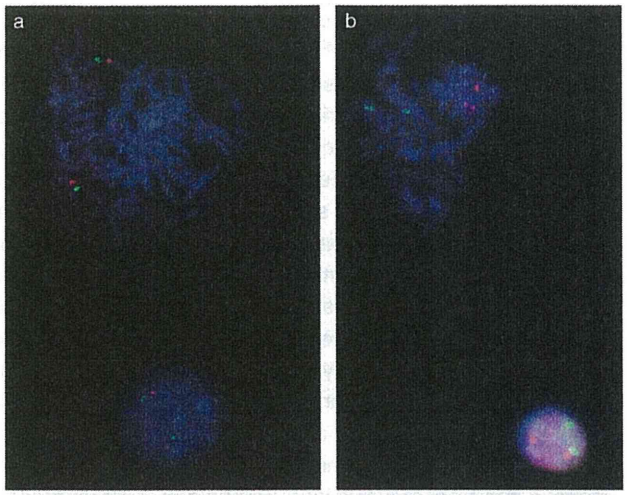

Figure 1 A metaphase spread (top) for testing a bacterial artificial chromosome (BAC) probe. (**a**) Two signals (green) with the corresponding centromere probe (red signals) are seen in the same chromosome. (**b**) Red and green signals are seen in different chromosomes, although they were supposed to be in the same chromosome according to the information in the database. Interphase cells exhibit two (pairs of) signals each (bottom).

Figure 2 Tissue microarray gauges, prefabricated recipient blocks with holes, commercially available, and embedded blocks from top to bottom. The core diameters are 3 mm, 2 mm, and 1 mm in diameter (left to right).

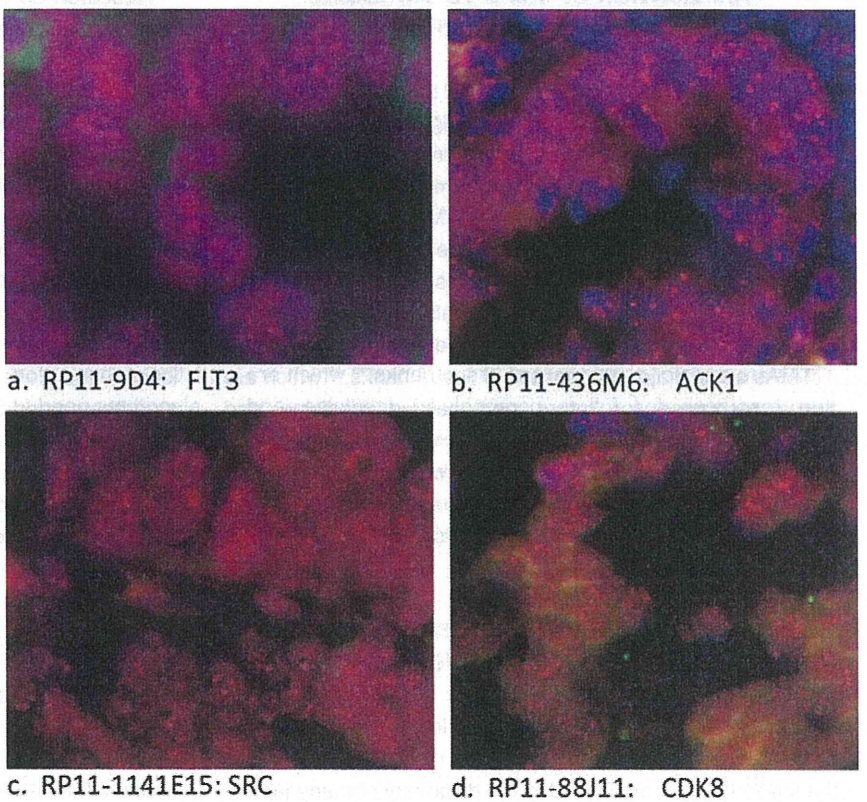

Figure 3 Amplification of kinase loci detected in FFPE tissues from the undifferentiated carcinoma of the stomach. Symbol genes are FMS related tyrosine kinase 3 (FLT3) (**a**), Activated p21CDC42 kinase(ACK1) (**b**), V-SRC avian sarcoma (Schimidt-Ruppin A-2) viral oncogene (SRC) (**c**), and Cyclin dependent kinase 8 (CDK8) (**d**). The probes were labeled with Spectrum Orange (Abbott, Abbott Park, IL, USA), and the nuclei were stained with 4, 6-diamino-2-phenyl indole dihydrochloride (DAPI, Abbot). The method is described in detail in the previous literature.

that must be overcome. Many investigators have constructed tissue microarrays for efficient use of probes they had laboriously prepared, especially in retrospective studies. The idea of embedding many pieces in a single block existed in the early days of anatomical pathology, but several embedding instruments for this purpose recently became popular, and technical refinements are under way. One well-circulating brand of microarray instruments is Beecher Instruments (Beecher Instruments, Inc. Sun Prairie, WI, USA). Their models have 0.6 mm, 1 mm, 2 mm cylinders, and the Azumaya model KIN-1 and model FIN-3 (Azumaya Cooperation, Tokyo) have wider cores that are 2 mm, 3 mm, 5 mm, and 7 mm in diameter. There are pros and cons in regard to using the smaller cores, and several problems encountered in using the instruments with various sized-cores are addressed in the instructions included with each of the instruments. A validation study in regard to possible sampling error when small core specimens are collected was performed and the results were published.[40] Very recently, donor blocks containing multiple slots and an apparatus for making them have become commercially available (Fig. 2) (Patent Application 2009-028167), and many other variations will become available commercially. In addition to genomic and immunohistochemical studies, a proteomics approach by imaging mass spectrometry on a TMA platform is also feasible.[41]

APPLICATION OF TMAS TO BIO-BANKS AND ETHICS CONSIDERATION

Preparations of TMAs and requests to prepare them will become more frequent in both investigative and diagnostic pathology laboratories, and as members of institutional review boards (IRBs) pathologists are sometimes responsible for appropriate control of these TMA bioresources. The categories of pathology specimens are described in several documents and on several websites,[42–44] and IRBs are required to facilitate research proposals of making or using TMAs to implement the research smoothly and ethically.

TMAs are a major component of tissue banks,[45] which are tissue resources for future personalized medicine and national and international bio-bank systems are now being established (websites: http://www.stn.org.sg, http://www.ukbiobank.ac.uk, http://www.bbmri.eu, and http://www.src.riken.go.jp/english/project/person/index.html).

COPY NUMBER ALTERATIONS DETECTED BY aCGH AND SNP ARRAY: USEFUL DATA FOR FISH ANALYSIS

Data on copy number alterations in solid tumors deposited in databases and publications have rapidly accumulated since the introduction of aCGH led to the discovery of many tumor-specific and stage-specific gains or losses of particular regions of chromosomes.[46–49] Much of the information generated by aCGH itself is used as a diagnostic or prognostic tool in pathology laboratories.[50–52] Information on genome-wide genetic changes in cancer DNA are now viewed as academic knowledge that is only useful to the graduate students and researchers, but sooner or later it will be an essential tool of the diagnostic pathologist facing daily challenges in diagnosis and management. There are many issues in conventional pathology research and practice to which human genome data can be applied.[53] Sano *et al.* conducted a chromosome-wide survey to the archives of adenomatous hyperplasia of the lung[38] and proposed 'adenocarcinoma in adenomatous hyperplasia' as an early stage of carcinogenesis of lung adenocarcinoma. Although the tools were genetic, the story they told was morphological. Very recently, a more powerful system, an SNP array platform containing more than 500 000 SNP sites has come into widespread use, and copy number estimation by several algorithms has facilitated identification of copy number changes, such as loss of heterozygosity, uniparental disomy, and amplification, in many clinical tumors. Midorikawa *et al.*[54] integrated the data based on pathological examination of 'nodule in nodule' in resected liver tissue with the results of a comprehensive copy number survey with the Affymetrix SNP array that were confirmed by FISH, and succeeded in clarifying genetic process in human hepatocarcinogenesis in detail.

Research on structural changes and balanced translocation of chromosomes in solid epithelial tumors is also a cutting edge area of research today.[7,51,55–57] The numbers of candidate probes that should be investigated for clinical significance seem huge. Several points need to be addressed when interpreting the results generated when an aCGH array and SNP array are used to analyze a human tumor genome. The first point is that many platforms are available to analyze copy number alterations, and a few papers on the characteristics of each platform have been published.[58–60] Furthermore, since many algorithms are available to enumerate copy numbers on the same platform, the characteristics of the platforms themselves and the benchmarks of the algorithms need to be known. Most algorithms for estimating the copy number of loci set the reference dosage of tumor autosomes as 2 (diplotype), but this reference number is not valid for most common epithelial malignant tumors. Ng *et al.*[61] recently refined the protocol for ploidy-specific copy number estimation, and obtained a better threshold for detecting CNA in cell lines, and Suzuki *et al.* performed a benchmark test of two widely used algorithms and extensively characterized the features of the algorithms in terms of different formulas for setting the gain or loss thresholds of genetic loci.[62] Because of the intrinsic limitations of each method, two or three methods need to be used simultaneously for the same tumor.

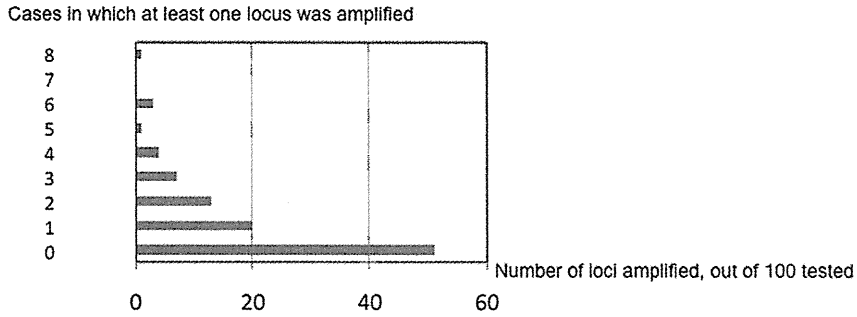

Figure 4 Distribution of the numbers of the loci amplified in any of the 60 cases (20 gastric cancer cases, 20 lung cancer cases, and 20 colon cancer cases) in a discovery set. More than half (51) of the 100 loci tested were not amplified in any of the 60 cases tested. Five or more loci were amplified in 5 (8%) of the 60 cases tested.

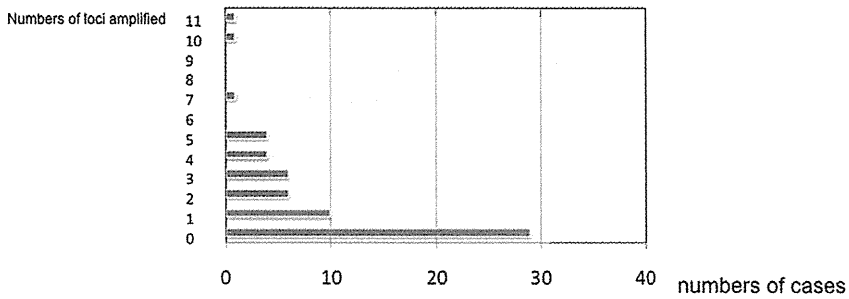

Figure 5 Distribution of cases according to numbers of loci amplified (vertical axis). From 0 to 11 of the 70 or more (as many as 100) loci successfully tested were amplified. None of the 100 loci were amplified in 29 of the cases. Seven or more loci were amplified in 3 cases.

WILL FISH BECOME A POPULAR AND ACCEPTED DIAGNOSTIC TOOL IN PATHOLOGY PRACTICE, ESPECIALLY IN GUIDING INDIVIDUAL CANCER THERAPY?

Only a few FISH kits have been authorized for clinical use, but many are available for use in research. Translocation detection kits are often used to confirm diagnoses.[63,64] Mori *et al.* recently used tens of BAC probes to make the differential diagnosis between adrenal tumors.[65] However, the clinical significance of copy number alterations warrants further accumulations of retrospective and prospective data. The rationale for the efficacy of molecularly targeted drugs varies with the mutation, overexpression, and genomic amplification of the target molecules, such as HER2 and EGFR. Fu *et al.* investigated copy number changes and expression of GATA-6 in pancreatic cancer and reported finding consistency between the results for overexpression and amplification of the genomic area of the GATA-6 locus,[66] and they also validated their findings observation by FISH. Amplification itself, however, does not always imply activation of the molecules or pathways of the genes on that genomic locus. Actually the EGFR immunohistological findings in lung cancer cells are not always consistent with the FISH data,[67] and borderline grades of immunostaining of HER2(2+) require FISH analysis to determine whether the HER2 gene has been amplified. Another receptor kinase gene, *MET*, has been evaluated as a potential target of tailor made therapy in the same manner as the *EGFR* gene and *HER2* gene have, and in some studies *MET* amplification has been found to predict shorter patient survival after surgical resection of non-small cell lung cancer.[68] Amplifications of PIK3CA is found in a considerable percentage of non-small cell lung cancers, and it and PIK3CA mutation are mutually exclusive.[69] The list of the amplified segments continues to increase, although validation of their clinical significance awaits further study. The list of tumors in which amplification of certain gene product(s)can be indentified has been growing, meaning that the list of the promising targets of therapy is also growing. Comprehensive copy number analysis by large-scale sequence technology has revealed that a copy number gain of an unexpectedly high proportion of genes that encode kinases in cancers.[11] We tested 100 BAC probes containing different kinase loci in a gastric, colorectal, and lung cancer detection sets (20 cases for each organ) by TMA-FISH technology, and found amplification of at least one kinase gene in a considerable number of cases, or, expressed another way, found that unexpected kinase loci were amplified in a significant proportion of human common solid tumors (Figs 3–5). The discovery blocks we used consisted of tumor tissues in both early and advanced stages, and various histological types. The observation above has also provided us with the following perspectives. Combinatory chemistry has already generated many drugs targeted to kinase genes or their products, thus amplifications of specific sites on certain kinase genes are amenable to pharmacological intervention which that will lead to the establishment of the target specific therapy. When observations like ours are validated and refined for clinical evaluation, the FISH diagnostic system with particular kinase probes may serve as another basis of tailor-made cancer therapy.

Major issues, however, remain to be resolved for before authorization of FISH-based diagnostic tools even if scientifically validated. Cost-benefit analysis of so-called targeted therapy is just starting in the tight-fisted health insurance environment, and there are gloom and doom forecasts that a bonanza of new authorized diagnostic kits is unlikely to arrive anytime soon. The time-line of the last few decades, however, in which many antibodies eventually became essential in pathology labs, evokes us a very different picture.

CONCLUSIONS

The basic knowledge required to perform the combination of TMA and FISH with many BAC probes is familiar to diagnostic pathologists, but that is different from actually running it (TMA-FISH with BACs) in real pathology practice. Obtaining an ample numbers of BAC probes, labeling, and expensive fluorescence microscopes may be hurdles for modestly equipped community hospitals. In the previous issue of *Pathology International,* Kato *et al.*[70] have reported their experience with using of a commercialized product that applies chromogenic *in situ* hybridization, a friendlier method that allows the use of ordinary microscope.

Many DNA probes labeled 'research use only' are actually used in sarcoma diagnosis,[20] and standardization and quality control of only a few FISH diagnostic systems have been achieved. Most of DNA probes are expensive, and there are few 'generic' diagnostic kits.

Over the coming decades, DNA probes will become a familiar diagnostic tool to the pathologists in community hospitals, and the information obtained by using them will suggest therapeutic guidance as well as the diagnosis. At the same time, the accumulation of the data generated by TMA-FISH approach will complement numerous *OMICS* data that have been accumulating in other disciplines of medicine. In other words, the TMA-FISH approach may be one of the smartest harvest (exit) strategies among *OMICS* projects related to human cancer, and many investments have been made in it over the last two decades.

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Esophageal Mucosal Injury With Low-Dose Aspirin and Its Prevention by Rabeprazole

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Aspirin is used widely as an antithrombotic drug for the prevention of cardiovascular and cerebrovascular events. Although aspirin increases the risk for gastrointestinal mucosal injury, the effect on esophageal mucosa is unclear. This study investigates whether aspirin induces esophageal mucosal injury and whether a proton-pump inhibitor can prevent such injury in relation to CYP2C19 genotypes. Fifteen healthy Japanese volunteers are dosed for 7 days in a 5-way randomly crossover trial: placebo, aspirin 100 mg, rabeprazole 10 mg, and aspirin 100 mg plus rabeprazole 10 mg either once daily or 4 times per day. All subjects undergo endoscopy and 24-hour intragastric pH monitoring on day 7. With the aspirin regimen, esophageal mucosal disorders occur in 7 patients (46.7%) (5, grade M; 2, grade A). The median 24-hour pH differs significantly among subjects who develop grade M or A gastroesophageal reflux disease and those who do not develop gastroesophageal reflux disease; the median pH in grade A gastroesophageal reflux disease is significantly lower (1.5 [range, 1.1-1.9]) than that in patients without gastroesophageal reflux disease (5.6 [range, 0.8-8.4], P = .04). Rabeprazole significantly inhibits acid secretion irrespective of CYP2C19 genotypes and decreases the incidence of aspirin-related esophageal injury and symptoms according to increasing pH value. Aspirin induces esophageal mucosal injury in an acid-dependent manner. Concomitant proton-pump inhibitor therapy may prevent advanced effects of low-dose aspirin.

Keywords: *Aspirin; nonsteroidal anti-inflammatory drugs; gastroesophageal reflux disease; rabeprazole; CYP2C19*

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Gastroesophageal reflux disease (GERD) is common, affecting around 20% to 30% of the population worldwide, but particularly in Western countries.[1] Major risk factors for the development of GERD are frequent and transient relaxation of the lower esophageal sphincter, impaired esophageal clearance of regurgitated gastric acid, frequent reflux of gastric contents, delayed gastric emptying, and the presence of hiatus hernia.[2,3] Nonerosive GERD (NERD) was established as a disorder in which patients complain of troublesome reflux-associated symptoms (ie, heartburn and epigastric discomfort) but do not have breaks in the esophageal mucosa (ie, erosion or ulcer) on endoscopy. Because of the increased incidence rate of both GERD and NERD, their physiological characteristics and treatment strategies are being intensively investigated.[4-6]

Usage of nonsteroidal anti-inflammatory drugs (NSAIDs) is increasing yearly with the aging of the population and the increased treatment of chronic arthritis and collagen diseases. Aspirin has an antiplatelet effect by decreasing the production of platelet thromboxane A2 via inhibition of cyclooxygenase-1 (COX-1) and is used widely as an antithrombotic drug.[7] NSAIDs are reported to induce esophageal mucosal injury, such as benign esophageal stricture, GERD, and esophageal ulceration.[1,7-12] Through its inhibition of the synthesis of gastrointestinal

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mucosal COX-1, which produces protective prostaglandins, aspirin increases the risk of developing peptic ulcer by 2- to 5-fold [13]. Aspirin is also associated with a significant risk for developing serious gastrointestinal complications even at a daily dose of 75 mg.[14,15] Furthermore, although enteric-coated aspirin has decreased mucosal damage in the short term,[16] it does not decrease the risk for hemorrhagic gastrointestinal events compared with noncoated aspirin.[17,18]

Gastric acid secretion plays an important role in the development of NSAID-associated peptic ulcers, so proton pump inhibitors (PPIs) (eg, omeprazole, lansoprazole, rabeprazole [RPZ], and pantoprazole) are now widely used as the first-line therapy for acid- and NSAID-related gastroduodenal diseases.[19] Esophageal mucosal damage is also related to the intra-esophageal and intragastric pH; to treat GERD, the duration of intragastric pH less than 4.0 during a 24-hour period should be no longer than approximately 2 to 4 hours (ie, the percentage time for intragastric pH <4.0 should be <16.7%).[20] However, whether aspirin damages the esophageal mucosa, as observed in the gastric and enteric mucosa; whether gastric acid is involved in the induction of mucosal damage by aspirin; and whether a PPI would prevent the development of aspirin-induced esophageal injury are unclear.

PPIs are mainly metabolized by CYP2C19. In poor metabolizers (PMs), plasma levels of PPI such as omeprazole and lansoprazole are markedly increased and the pharmacodynamic effects of the PPI are enhanced in comparison with intermediate metabolizers (IMs) or rapid metabolizers (RMs) of CYP2C19.[21-23] In fact, the cure rate of GERD by lansoprazole depends on CYP2C19 genotype status, and that of PMs is highest.[24] RPZ, on the other hand, is reduced mainly via a nonenzymatic pathway,[25,26] and therefore its effect on acid secretion is assumed to be less influenced by CYP2C19 genotype than for the other PPIs.[21,27] However, several studies have revealed that the pharmacokinetics and pharmacodynamics of RPZ depend on CYP2C19 status, but whether the preventive effect of RPZ on aspirin-induced esophageal mucosal injury depends on CYP2C19 genotype is unclear.

To our knowledge, no previous studies have prospectively investigated whether low-dose aspirin induces esophageal mucosal injury according to gastric acid secretion and whether such injury can be prevented by the acid-inhibiting effect of a PPI, especially as related to CYP2C19 genotype status. We aimed to investigate both the histopathologic and endoscopic effects of low-dose aspirin on the esophageal mucosa and the characteristics of patients who developed GERD or NERD with low-dose aspirin therapy. We also studied the role of gastric acid secretion and its inhibition by a PPI in relation to different CYP2C19 genotypes.

Table I Demographic Characteristics of *Helicobacter pylori*–Negative Volunteers Between Patients With and Without Endoscopically Determined Esophageal Damage in Aspirin-Alone Regimen

	With GERD	Without GERD	*P* Value
GERD grade, grade M/grade A, n	5/2	—	
Age, y, mean ± SD	20.4 ± 0.7	20.8 ± 0.2	.17
Sex, male/female, n	6/1	4/4	.74
Body weight, kg, mean ± SD	54.0 ± 2.6	57.6 ± 4.0	.52
PG I, ng/mL, mean ± SD	46.8 ± 6.0	46.9 ± 2.1	.96
PG II, ng/mL, mean ± SD	8.9 ± 1.0	8.0 ± 0.2	.79
PG I/PG II ratio, mean ± SD	5.5 ± 0.1	5.8 ± 0.3	.50
CYP2C19, RM/IM/PM, n	2/4/1	3/1/4	.15
Symptom, median (range)			
Epigastric discomfort	1 (1-4)	1 (1-1)	.35
Heartburn	2 (1-5)	1 (1-2)	.27
Hernia, presence/absence, n	6/1	4/4	.14

GERD, gastroesophageal reflux disease; PG, pepsinogen; RM, rapid metabolizer; IM, intermediate metabolizer; PM, poor metabolizer.

MATERIALS AND METHODS

Subjects

After providing written informed consent, 58 healthy young Japanese volunteers underwent serological testing for *Helicobacter pylori* infection (HM-CAP kit, Enteric Products, Stony Brook, NY), upper endoscopy, and CYP2C19 genotyping. Subjects with *H pylori* infection, with endoscopically esophageal mucosal injury, and with GERD-related symptoms were excluded to avoid modification of *H pylori* infection and GERD. From the remaining subjects, 15 subjects (5 RM, 5 IM, and 5 PMs) were randomly selected and invited to participate in the study (Table I). Serum pepsinogen (PG) I and PG II levels

were measured in all 15 subjects by radioimmunoassay (Abbott Japan, Tokyo, Japan), and the ratio of PG I/PG II was calculated as a serological evaluation of atrophic gastritis.[28]

We choose sample size by reconciling the same sample size involved in previous reports,[23,29,30] because significant differences in pharmacodynamic parameters of PPIs among different CYP2C19 genotype groups, where the sample size was around 15 subjects (5 subjects for each genotype group), were demonstrated.

Study Protocol

All subjects were randomly dosed with 5 regimens including a placebo and the 4 different drug regimens: (1) RPZ (Pariet, Eisai, Tokyo, Japan) 10 mg once daily dosing (RPZ-10), (2) aspirin (Bayer, Osaka, Japan) 100 mg once daily dosing (ASP), (3) RPZ 10 mg once daily dosing plus aspirin 100 mg once daily dosing (ASP-RPZ-10), and (4) RPZ 10 mg 4 times daily plus aspirin 100 mg once daily dosing (ASP-RPZ-40). On day 7 of each of the 5 treatment regimens, upper gastroduodenal endoscopy and 24-hour intragastric pH monitoring were performed. All subjects were provided with 3 meals each day (breakfast at 8 AM, lunch at 12:30 PM, and supper at 6 PM). Mineral water was allowed ad libitum, but no other beverages (eg, grapefruit juice) were permitted. There was a washout period of at least 2 weeks between the 2 study periods, and we checked whether symptoms had disappeared after the end of the previous regimen. All protocols of each volunteer were finished within 6 to 10 months. None of the subjects had consumed alcohol or had a smoking habit. None had taken any drugs for at least 1 week prior to the study, nor did they take any during the study.

The protocol was approved in advance by the Human Institutional Review Board of the Hamamatsu University School of Medicine. Written informed consent was again obtained from each subject before participation in each of the 5 trial phases.

Gastroduodenal Endoscopy and Pathological Findings of the Esophagus

On day 7 of each trial phase, upper gastroduodenal endoscopy was performed by only one endoscopist (MN, who was unaware of any clinical information regarding the subjects) in all subjects after overnight fasting, and the esophagus was assessed according to the Los Angeles classification (grade A to D).[31] Grade M was assessed as a mucosal finding with redness, edema, or white-granules in the endothelial cell (EC) junction, and grade N was normal mucosa in patients with acid reflux-related symptoms, such as heartburn. Later, a total of 3 endoscopists finally diagnosed the grade of GERD by using video. Biopsy specimens were taken from the esophageal mucosa at 1 cm above the EC junction to investigate aspirin-induced mucosal changes without erosion.

The esophageal biopsy specimens were stained with hematoxylin–eosin, and the degree of inflammation was assessed according to the updated Sydney system.[32] Although the updated Sydney system was validated for gastritis but not for reflux esophagitis, this system was substituted as the scoring system of esophageal mucosal inflammatory cell infiltration level. The grade of dilatation of small blood vessels was assessed as follows: grade 0, normal (<2 red blood cells [RBCs] in small blood vessels); grade 1, slight dilatation (2-4 RBCs); or grade 2, moderate to severe dilatation (>4 RBCs). A single pathologist (HS), who was unaware of any clinical information regarding the subjects, examined all the biopsy specimens.

24-Hour Intragastric pH Monitoring

On day 7 of each trial phase, approximately 1 hour after endoscopy, an antimony pH catheter (Medtronic, Minneapolis, Minnesota) was inserted transnasally under local anesthesia, placed 5 cm distal to the gastric cardia, and left in situ for 24 hours. The intragastric pH data were recorded with a Digitrapper pH 400 (Medtronic Functional Diagnostic A/S, Skovlunde, Denmark).

Although the measurement of intra-esophageal pH rather than intragastric pH may be suitable for investigation of esophageal injury by aspirin, the intragastric pH values were measured instead of intra-esophageal pH because intra-esophageal pH value was correlated with intragastric pH.[20]

CYP2C19 Genotyping

DNA was extracted from each subject's leukocytes using a commercially available kit (IsoQuick, ORCA Research, Seattle, Washington). Genotyping to identify the CYP2C19 wild-type (*1) gene and the 2 mutated alleles, *CYP2C19**2 (*2) in exon 5 and *CYP2C19**3 (*3) in exon 4, was performed by polymerase chain reaction-restriction fragment length polymorphism with allele-specific primers.[33,34] CYP2C19 genotypes were classified into 3 groups: RM (**1/*1*), IM (**1/*2* or **1/*3*), and PM (**2/*2, *3/*3,* or **2/*3*).

Table II Intragastric pH With Different Regimens as a Function of CYP2C19 Genotype Status

	RM (n = 5)	IM (n = 5)	PM (n = 5)	*P* Value
Median intragastric pH				
Placebo	2.3 (1.5-2.5)	1.6 (0.9-2.5)	1.4 (1.2-2.1)	.11
Aspirin	2.5 (0.8-3.3)	2.3 (1.1-2.8)	1.9 (1.1-2.8)	.65
RPZ 10 mg	5.6 (3.9-5.7)	5.2 (4.7-6.0)	5.9 (4.2-6.2)	.34
Aspirin + RPZ 10 mg once daily	5.4 (3.4-5.9)	5.0 (4.5-5.7)	6.0 (5.5-6.8)	.06
Aspirin + RPZ 10 mg 4 times daily	7.0 (6.2-8.0)*	6.4 (6.1-7.5)*	7.6 (7.3-8.4)*	.05
Median percentage of pH <4.0				
Placebo	86.3 (82.1-93.7)	92.9 (89.7-96.7)	93.2 (87.7-98.1)	.07
Aspirin	82.2 (67.1-98.1)	90.4 (76.5-99.0)	86.5 (78.4-95.6)	.77
RPZ 10 mg	33.2 (29.8-59.7)	39.6 (18.8-41.9)	24.6 (10.9-35.8)	.15
Aspirin + RPZ 10 mg once daily	37.7 (31.6-67.2)	39.0 (35.3-50.5)	32.2 (8.9-37.8)	.11
Aspirin + RPZ 10 mg 4 times daily	3.0 (0.0-21.7)*	6.9 (3.1-14.0)*	0.0 (0.0-0.3)*	.02

RM, rapid metabolizer; IM, intermediate metabolizer; PM, poor metabolizer; RPZ, rabeprazole. Values given as median (range).
*$P < .05$ (vs RPZ 10-mg regimen).

Symptom Index

The symptom index of heartburn and epigastric discomfort was assessed by visual analog scale as follows: grade 0 (no symptoms during dosing of drugs) to grade 7 (severe: several times per day every day during drug administration).

Data Analysis

The differences in the pathological and symptom scores among the different regimens were determined by Wilcoxon signed rank test, when significant differences were obtained by the Friedman test. Statistical differences in the median parameters for pathology, symptoms, and gastric acid secretion among the different endoscopic findings and among the 3 CYP2C19 genotypes were determined by Mann-Whitney *U* test, when a significant difference was obtained by the Kruskal-Wallis test. All *P* values were 2-sided, and $P < .05$ was taken to indicate statistical significance.

RESULTS

There were no severe adverse events, such as gastrointestinal hemorrhage and perforation, with any of the treatment regimens, which were tolerated by each subject irrespective of aspirin use with or without RPZ or CYP2C19 genotype.

Induction of GERD and NERD by Low-Dose Aspirin

With the ASP regime, 7 patients (47%) developed endoscopic esophageal mucosal injury (5 [33%] grade M and 2 [14%] grade A; Tables I and II). There were no statistically significant differences in clinical characteristics, such as age, body weight, serum PG I level, or PG I/PG II ratio, between the subjects who developed esophageal mucosal injury (GERD-positive group) and those did not (GERD-negative group) when aspirin 100 mg was dosed (Table I). Of the 15 subjects who did not previously have gastric acid reflux–related symptoms regardless of short-segment Barrett's esophagus, 5 (33%) experienced GERD-related symptoms of heartburn and/or epigastric discomfort more than once per week with the ASP regime (Table I).

24-Hour Intragastric pH Profiles for the Different Treatment Regimens

The median intragastric pH–time profiles throughout 24 hours were fairly similar between the placebo and aspirin-alone regimens and between the RPZ-10 and ASP-RPZ-10 regimens (Figure 1). The median pH–time profile for the ASP-RPZ-40 regimen was the highest compared with the other regimens.

In the RPZ-10 and ASP-RPZ-10 regimens, the median pH values were 5.6 (range, 3.9-6.2) and 5.5 (3.4-6.8), respectively, which were significantly higher than those for the placebo (1.6 [0.9-2.5], $P < .01$) and ASP regimens (2.1 [0.8-3.3], $P < .01$) (Figure 2A). In the ASP-RPZ-40 regimen, the median pH (7.3 [6.1-8.4]) was significantly higher than that with the ASP-RPZ-10 (Figure 2A).

In the RPZ-10 and ASP-RPZ-10 regimens, the median percentage times of pH less than 4.0 were 35.0% (10.9%-59.7%) and 37.0% (8.9%-67.2%), respectively, which were significantly higher than

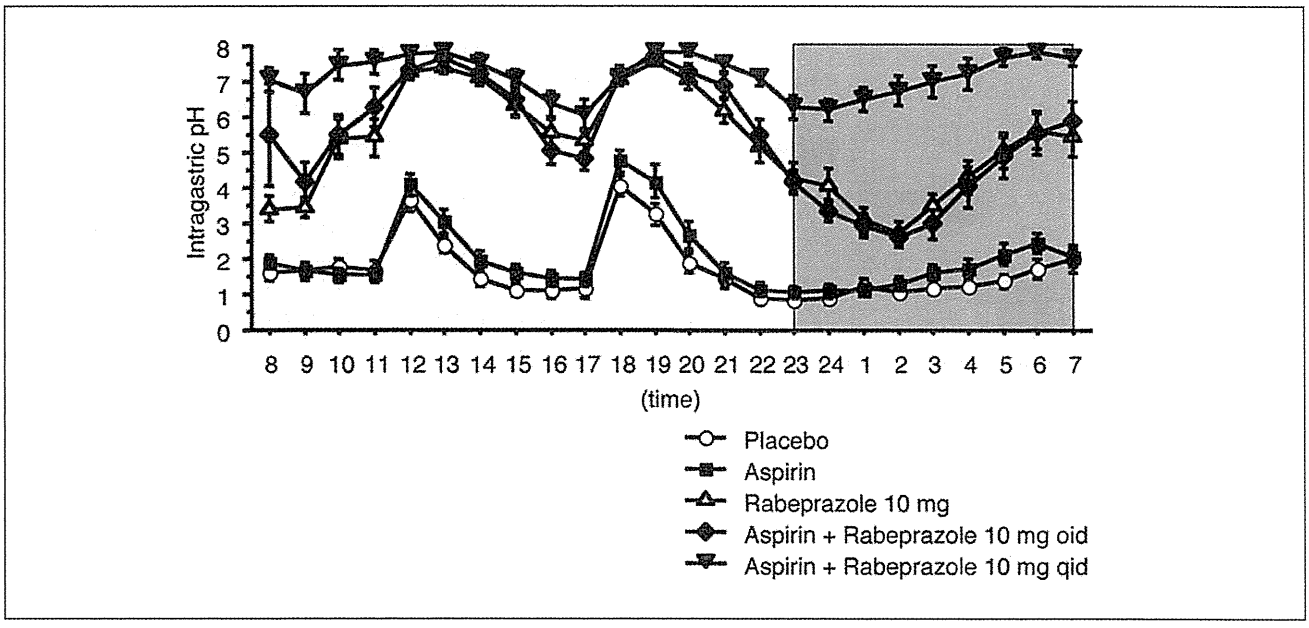

Figure 1. The median 24-hour pH-time profiles on day 7 of 5 different treatment regimens. The pH profile of RPZ 10 mg 4 times daily plus aspirin regimen was highest, those of RPZ 10 mg once daily dosing and RPZ 10 mg plus aspirin come next, and those of aspirin alone and placebo were lowest.

those for placebo (91.9% [82.1%-98.1%], $P < .01$) and ASP (89.1% [67.1%-99.0%], $P < .01$) (Figure 2B). In the ASP-RPZ-40 regimen, the median percentage time of pH less than 4.0 (3.0% [0.0%-21.7%]) was significantly higher than that with the ASP-RPZ-10 (Figure 2B).

Influence of CYP2C19 Genotype on 24-Hour Intragastric pH Profile and the Preventive Effect of RPZ on Aspirin-Induced Esophageal Mucosal Injury

The median pH and the median percentage time of pH less than 4.0 in the RPZ-10 regime did not differ significantly among the different CYP2C19 genotype groups (Table II). In the ASP-RPZ-40 group, the median pH and percentage time of pH less than 4.0 differed significantly; the median pH was higher and the median percentage time of pH less than 4.0 was lower in PMs than in RMs and IMs ($P < .05$) (Table II). In each CYP2C19 genotype, the median pH with ASP-RPZ-40 significantly increased compared with the ASP-RPZ-10 regime ($P < .05$) (Table II). There was no statistically significant difference in the preventive effect of RPZ-10 on aspirin-induced esophageal mucosal injury among the 3 different CYP2C19 genotypes (data not shown).

Table III Endoscopic Finding (Los Angeles Classification) by Different Treatment Regimens

Treatment Regimens	Normal	Grade M	Grade A	*P* Value
Placebo	15 (100)	0 (0)	0 (0)	.04
RPZ 10 mg	14 (93)	1 (7)	0 (0)	
Aspirin	8 (53)	5 (33)	2 (14)	
Aspirin + RPZ 10 mg once daily	14 (93)	1 (7)	0 (0)	
Aspirin + RPZ 10 mg 4 times daily	14 (93)	1 (7)	0 (0)	

Values given as n (%). *P* value measured the differences of endoscopic finding in each treatment regime.

Relationship Between Intragastric pH and Aspirin-Induced Esophageal Injury in Relation to RPZ Dosage

The incidence of esophageal mucosal injury in ASP-RPZ-10 and ASP-RPZ-40 decreased to 6.7% (1/15, grade M) and 6.7% (1/15, grade M), respectively, according to the increase in the 24-hour pH and the decrease in percentage time of pH less than 4.0 (Table III). The incidence of GERD-related symptoms more than once per week also decreased to 20% with the combined regimen.

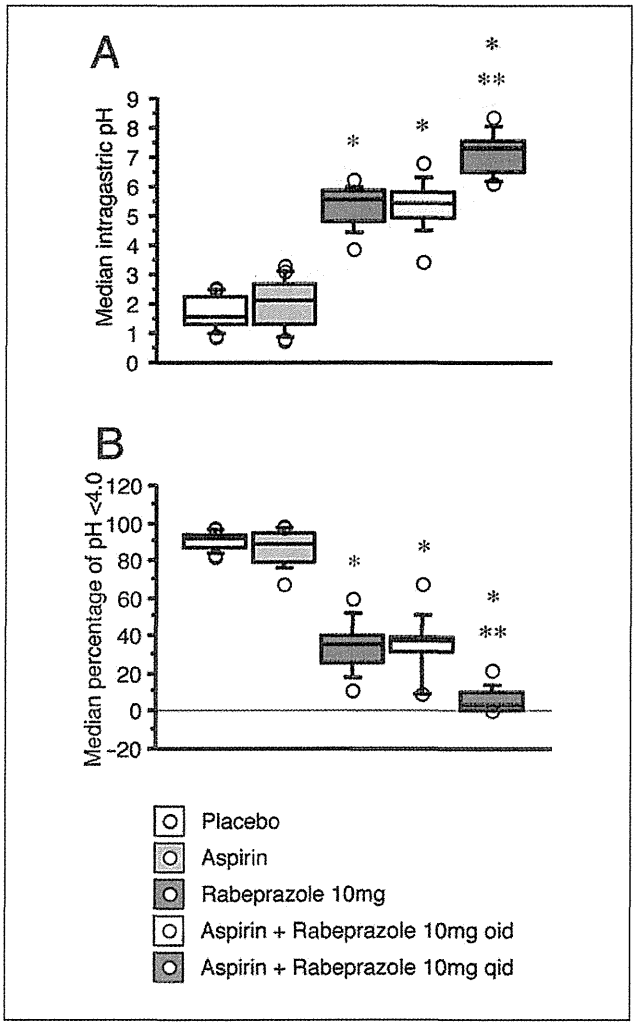

*Figure 2. The 24-hour median intragastric pH values (A) and the median percentage of intragastric pH less than 4.0 (B) in 5 different treatment regimens. *P < .05 (vs placebo) and **P < .05 (vs RPZ alone).*

When all pH data of aspirin-contained regimen irrespective of concomitant RPZ dosing were analyzed, the 24-hour pH profiles differed among the groups with normal mucosa as determined endoscopically (5.6 [0.8-8.4]), NERD grade M (2.6 [1.0-7.9]), and GERD grade A (1.5 [1.1-1.9]) (Figures 3A and 3B). The median pH in the GERD grade A group was significantly lower than that of the normal mucosa group ($P = .04$). The median percentage time of pH less than 4.0 in the GERD grade A group was 90.3% (86.5%-94.0%), which was significantly longer than that in the normal group (33.4% [0.0%-98.0%], $P = .04$) (Figure 3C).

When values were divided into nighttime (11 PM to 7 AM) and daytime (7 AM to 11 PM), the median daytime pH values in the ASP and ASP-RPZ-10 regimens irrespective of the grade of esophageal mucosal injury were significantly higher than those for the nighttime (Figures 4A and 4B). However, there was a significant difference between the GERD grade A group and normal group in the median nocturnal pH and the median percentage time of a nocturnal pH less than 4.0 (both $P = .04$) (Figures 4A and 4C). However, the median pH and the median percentage of time pH less than 4.0 in the daytime were not significantly different among the different GERD groups.

When subjects were classified into 2 groups with or without GERD-related symptoms (ie, heartburn), the median pH in the group without heartburn appeared higher than that in the group with heartburn but was not statistically significant (Figure 5A). Similarly, the median percentage time for intragastric pH less than 4.0 was not significantly different between the 2 groups (Figure 5B).

Pathological Findings During Aspirin Intake

Although the finding of more than 1 site of inflammatory cell infiltration and dilatation of small blood vessels was more often observed in biopsies from the ASP group compared with ASP-RPZ-10, there were no statistically significant differences in inflammatory cell infiltration and dilatation of vessels among the different regimens (Table IV). When subjects were divided into endoscopically normal, NERD grade N, NERD grade M, and GERD grade A, the finding of more than 1 site of inflammatory cell infiltration and dilatation of vessels occurred more often in subjects who developed NERD grade M or GERD grade A (Table V).

DISCUSSION

In this study, we demonstrated that 7-day treatment with low-dose aspirin caused esophageal mucosal injury in about half of healthy young subjects. We also found that the occurrence and prevention of esophageal mucosal injury induced by low-dose aspirin were closely related to intragastric pH values, especially at nighttime, and that the Japanese standard dose, 10 mg of RPZ, could effectively prevent the low-dose aspirin-induced esophageal mucosal injury. On the other hand, low-dose aspirin had no significant influence on intragastric pH value.

Recent increases of aspirin use and occurrence of GERD have suggested a possible role of low-dose of aspirin in the development of GERD in aspirin users.