COMMENTARY

Telomerase and Early Detection of Cancer: a National Cancer Institute Workshop

Rosalind A. Breslow, Jerry W. Shay, Adi F. Gazdar, Sudhir Srivastava*

Cellular immortality is a hallmark of cancer. The recent identification of telomerase, an enzyme associated with cellular immortality, at multiple points along the continuum of multistep carcinogenesis has created considerable interest in its potential for the early detection of carcinogenesis (1,2).

Telomerase is the enzyme that rebuilds telomeres, the TTAGGG-repeated ends of chromosomes. Germline cells express telomerase and are immortal. Although most fetal somatic cells express telomerase, the enzyme is considerably less active or inactive when the cells mature (3). Thus, most somatic cells do not express telomerase and are mortal. Because of an end replication problem, i.e., incomplete synthesis of the lagging strand during DNA replication, the telomeres of mortal cells shorten with age and eventually reach a critical point at which chromosomal integrity is lost and proliferation ceases (1). A rare somatic cell occasionally escapes programmed senescence by activating telomerase. The extended survival of such a cell could be conjectured to provide opportunities for the accumulation of additional genomic insults that might contribute to a progressive neoplastic evolution within that cell. If so, telomerase might serve as a key molecular marker of that process.

The goal of early detection is to identify potentially carcinogenic or frankly carcinogenic lesions as early in multistep pathogenesis as possible (4,5). Because the early detection of cancer is a dynamic process driven by continually advancing insights into the biology of carcinogenesis and both driven and limited by the availability of technologies, the meaning of ‘‘early’’ is necessarily in constant flux. The nature of an ideal early detection biomarker, however, is unchanging; it should be expressed at a stage that allows interventions of greater efficacy or less toxicity than would be possible if it were detected later.

Because telomerase might have potential as a biomarker for the early detection of cancer, the National Cancer Institute (NCI) convened the ‘‘International Workshop on Telomerase in the Early Detection of Cancer’’ on June 6-7, 1996, in Bethesda, MD. The purpose of the workshop was to determine what is known about telomerase and what direction future research should take.

In a keynote address, R. D. Klausner (Director, NCI) indicated NCI’s continuing interest in research on telomere biology. B. S. Kramer (Deputy Director, Division of Cancer Prevention and Control, NCI) asked the panelists, experts in telomere and telomerase research, to consider the following questions related to the validity of telomerase as a biomarker for the early detection of cancer: What is the differential expression of telomerase among normal tissues and organs? Is telomerase present early in the multistep pathogenesis of cancer, and is it a sensitive and specific marker for preinvasive neoplasia? During their deliberations, the panelists raised a related question: Does telomerase predict the prognosis of cancer patients? The panelists also considered other central questions: Is some manifestation of telomerase, such as its RNA component or telomere length, more closely associated with the early detection of cancer than the enzyme itself, and can a reliable test for telomerase that is non-invasive or minimally invasive and easily administered by clinicians be developed?

These questions and the panelists’ responses are discussed below.

Workshop Summary

What Is the Differential Expression of Telomerase Among Normal Tissues and Organs?

Some normal renewal tissues express low levels of telomerase. These low levels of the enzyme compensate only partially for the end replication problem responsible for the progressive shortening of telomeres with age (1). It is interesting that, in those renewal tissues that have detectable telomerase, telomeres continue to shorten throughout life, but perhaps at a slower rate.

The question of differential expression is particularly important in determining whether the enzyme has sufficient specificity to be useful in the early detection of cancer within various target tissues. If a sample of nondiseased tissue contains a mixture of cell types, some of which normally express the enzyme, an individual might be incorrectly identified as diseased. Therefore, it is important to determine the levels of telomerase that are normal for each tissue or organ, accounting for the telomerase contributed by background expression, including normally present tissue components. The panelists presented data indicating that telomerase expression is considerably lower in normal renewal tissues than in cancerous tissues.

*Affiliations of authors: R. A. Breslow (Applied Research Branch), S. Srivastava (Early Detection Branch), Division of Cancer Prevention and Control, National Cancer Institute, Bethesda, MD; J. W. Shay (Department of Cell Biology), A. F. Gazdar (Department of Pathology, Hamon Cancer Center), The University of Texas Southwestern Medical Center, Dallas.

Correspondence to: Sudhir Srivastava, Ph.D., M.P.H., National Institutes of Health, Executive Plaza North, Rm. 330, Bethesda, MD 20892.

See ‘‘Notes’’ following ‘‘References.’’

© Oxford University Press
Several workshop panelists reported that telomerase in the blood can be derived from mature cells as well as from stem cells. K. Hiyama (Hiroshima University School of Medicine, Japan) and S. Bacchetti (McMaster University Medical Center, Hamilton, ON, Canada) reported that normal peripheral mononuclear cells express telomerase, but at extremely low levels (i.e., about 1%-2% of the amount found in immortalized cancer cell lines) (6,7). J. Ohyashiki (Tokyo Medical College, Japan) similarly reported that normal mononuclear cells express low levels of telomerase and that enzyme expression decreases with age (personal communication). C. Greider (Cold Spring Harbor Laboratory, NY) reported that resting T cells express low levels of telomerase that increase as cells progress in the cell cycle (8). Her laboratory is currently conducting experiments to pinpoint the exact time of activation in G1.

Several panelists presented data on levels of telomerase in the gastrointestinal tract. E. Hiyama (Hiroshima University School of Medicine) detected telomerase in normal intestinal mucosa, but at levels 10-100 times lower than in cancerous mucosa (9); he concluded that the enzyme might originate from intestinal basol stem cells. H. Tahara (Hiroshima University School of Medicine) also reported finding low levels of telomerase in normal gastrointestinal mucosa (personal communication). J. Shay (The University of Texas Southwestern Medical Center, Dallas) suggested that the enzyme might originate from stem cells in the lower third of intestinal mucosal crypts or from inflammatory cells in the submucosa. A. Gazdar (The University of Texas Southwestern Medical Center) and co-workers (1) found telomerase in benign lymph nodes obtained from patients with lung cancer. The telomerase was probably derived from normal, mature lymphocytes. Gazdar noted that careful histopathologic evaluation is essential when clinical samples are assayed for telomerase.

Is Telomerase Present Early in the Multistep Pathogenesis of Cancer?

As noted previously, the “early” in early detection is a dynamic descriptor; its meaning will inevitably change as biology and technology advance. At present, “early in the multistep pathogenesis” is probably best defined by the histopathologic designation of preinvasive neoplasia. However, it can also be used to connote early invasive cancer. If telomerase could be used to detect a given stage of preinvasive neoplasia and that stage of carcinogenesis could be treated with greater efficacy and lesser toxicity than a later stage of the process, early detection based on telomerase might have the potential to reduce cancer morbidity and mortality. Likewise, if telomerase could be used to identify invasive neoplasia earlier than is currently possible, it might have an impact on cancer mortality, assuming effective treatment is available. Several panelists discussed the occurrence of telomerase in preinvasive and invasive neoplasia in tissues and organs. In addition, some presented data on telomerase expression in samples acquired from individuals by use of noninvasive or minimally invasive techniques. The degree of discomfort that an individual experiences in contributing biologic material to assay for telomerase is of particular relevance for screening. Screening, whether of asymptomatic individuals or individuals at risk, depends in large part on the willingness of individuals in the target population to contribute samples.

Preinvasive neoplasia in tissue and organ samples. The panelists reported that telomerase is expressed in preinvasive neoplasia of the colon, head and neck, and lung and that expression parallels histopathologic grade. Tahara reported detecting telomerase in about half of surgically obtained colon adenomas (10). Telomerase activity was weaker in these precancerous lesions than in samples of colon carcinomas. D. Sidransky (The Johns Hopkins University, Baltimore, MD) reported that about half of preinvasive oral lesions, primarily dysplasias and carcinomas in situ, expressed telomerase (11). Gazdar and co-workers (1) demonstrated that telomerase is expressed in microdissected epithelia in preinvasive lung neoplasia and that expression increases progressively with histopathologic grade. Using bronchial tissues obtained at surgery or bronchoscopy from 15 patients, this group of investigators detected telomerase in about one fourth of histologically confirmed hyperplastic tissues, one third of dysplastic tissues, and all carcinomas in situ. Telomerase activity was considerably weaker in these preinvasive lesions than in the corresponding invasive tumors.

Neoplasia in tissues and organs. The panelists also reported that telomerase is expressed in brain cancer and leukemia and in carcinomas of the colon and head and neck. L. Langford (The University of Texas M. D. Anderson Cancer Center, Houston) studied telomerase activity in brain tumors (12). Almost all of the malignant and atypical meningiomas from 52 patients expressed telomerase, while only about half of the ordinary (morphologically benign) meningiomas expressed the enzyme. D. Tarin (University of Oxford, U.K.) reported on telomerase activity in colon cancer (13). He and his group found that the majority (95%) of 35 surgically obtained colon carcinoma samples expressed telomerase; in contrast, nonmalignant samples showed only weak expression of the enzyme in 14% of 35 matched samples. Tahara also found that a high proportion of colon carcinomas expressed telomerase (10,14). Sidransky studied telomerase activity in head and neck carcinomas and reported that more than three fourths expressed the enzyme (11). Ohyashiki studied telomerase activity in leukemia (personal communication). About three fourths of 55 patients with acute myeloid leukemia and about three fourths of 23 patients with acute lymphoid leukemia expressed telomerase. W. Zhang (The University of Texas M. D. Anderson Cancer Center) also studied telomerase activity in leukemia (15). He reported that about three fourths of 56 patients with acute myeloid leukemia had elevated telomerase activity and that the highest activity occurred in patients with chromosome 11q abnormalities and deletions on chromosomes 5 and 7 (unfavorable cytogenetics). K. Hiyama reported that all 11 surgically resected specimens of primary small-cell lung cancer from 11 patients had high levels of telomerase, whereas activity ranged from undetectable to high levels in 125 specimens of primary non-small-cell lung cancers from 125 patients (16). E. Hiyama studied pancreatic cancer (personal communication). Telomerase was detected in 41 of 43 malignant pancreatic neoplasms but not in any of 11 benign lesions.

Invasive cancer detected by use of noninvasive or minimally invasive sampling. Because telomerase may have potential as a screening tool, it is important to determine whether it can be measured in samples acquired from individuals with relative ease, i.e., without invasiveness or with minimal invasive-
ness. It is also important to validate telomerase in these samples against a ‘‘gold standard’’ with high laboratory validity, i.e., to determine the screening validity of telomerase.

Tarin studied an unselected, consecutive series of patients, many of whom had very early or minimal disease, to assess whether telomerase might have potential screening validity in bladder, breast, and colon cancers (13,17,18). Exfoliated bladder cells were obtained from a single 50-mL sample of urine from patients with and without bladder cancer (17). Telomerase was detected in approximately 16 of 26 patients with cancer but in only three of 83 individuals without cancer, resulting in a sensitivity of less than 7%, a specificity of 96%, and a positive predictive value of 84%. In patients with and without breast cancer, fine-needle aspirate samples also yielded a sensitivity of 67%, a specificity of 90%, and a positive predictive value of 77% (18). Colonic washings from a hospital-based population of patients with and without colon cancer resulted in a sensitivity of 60%; specificity and positive predictive values were both 100% (13). E. Hiyama detected telomerase activity in 100% of fine-needle breast aspirates obtained from 14 patients who, upon subsequent surgery, were diagnosed with histopathologically confirmed breast cancer (19). Among surgically resected gross samples, telomerase was detected in 130 (93%) of 140 breast cancers. K. Hiyama reported that cytologic samples obtained from lung cancer patients by flexible fiberoptic bronchoscopy expressed telomerase (20), and Sidransky found that telomerase expression in oral rinses, snap-frozen and stored up to 2 years, was associated with the presence of head and neck cancer (11).

Does Telomerase Predict Prognosis in Cancer Patients?

Although this meeting focused on early detection, panelists also presented information about the potential of telomerase to predict clinical outcome in patients with brain, breast, and head and neck cancers and leukemia. Langford reported that telomerase activity was significantly associated with poor prognosis in patients with telomerase-positive ordinary meningiomas and noted that the presence of telomerase activity in these morphologically benign, ordinary meningiomas is clinically relevant because it suggests that these tumors may contain a population of immortal cells (12). Ohyashiki noted that acute leukemia patients with the highest telomerase levels had the worst prognosis and that telomerase activity decreased during remission (personal communication).

Panel Discussions

At the conclusion of the workshop, the panelists discussed issues in the following three areas that are central to continued progress in telomerase research: molecular technology, basic biology, and clinical and translational applications. The discussions underscored the new and exploratory nature of research in this field.

Molecular Technology

The panel, which included scientists from industry who are actively working on the development of telomerase assays, discussed the possibility that some manifestation of telomerase, such as its RNA component (human telomerase RNA, also called hTR), might be more closely associated with the presence of preinvasive or invasive neoplasia than the enzyme itself. The panelists also discussed the merits of different assays. Greider suggested that the utility of a particular assay may vary by cancer site and by the end point being studied; i.e., while the results of one type of assay may correspond well with the presence of neoplasia, another may provide a better prediction of clinical prognosis or outcome. N. W. Kim (Geron Corporation, Menlo Park, CA) noted that hTR is theoretically the most sensitive assay for detecting telomerase. He also noted that the optimal assay chosen depends on the type of sample available. For example, telomerase activity is lost when tissues are fixed; therefore, the telomeric repeat amplification protocol (TRAP) assay (21), a polymerase chain reaction-based assay used by many investigators to determine telomerase activity in fresh or frozen samples, cannot be used on fixed, clinical specimens such as those routinely obtained from surgical specimens. The need to standardize assays was also discussed. Kim suggested that ribosomal RNA might be useful. Shay stated that, at present, the primary focus of molecular technology should be on further elucidating the nature and utility of hTR and on the development of in situ assays, i.e., assays that can be conducted on fixed, clinical tumor samples for which archived specimens are readily available.

Shay noted that the technology for measuring telomerase will advance rapidly and suggested the establishment of repositories containing paired neoplastic–non-neoplastic adjacent tissues so that different groups can compare new technologic advances against a standardized panel of samples. Frozen samples could be used to evaluate new measurement methodologies as well as to conduct prospective and nested case–control studies. As knowledge of the biology of telomeres and telomerase continues to evolve, it is likely that technologies will become available to quantitatively measure this enzyme, or some manifestation thereof, in a standardized (preferably automated) manner.

Basic Biology

The panelists discussed various aspects of telomere length. Bacchetti stated that some cancer cells may attain immortality using bypass mechanisms independent of telomerase. Greider noted that factors regulating telomere length, other than telomerase, have not yet been identified in human cancer cells but have been identified in yeast cells. Regarding the utility of telomere length in early detection, E. Hiyama stated that telomere lengths were similar in 50% of neoplasms and histopathologically normal adjacent tissues in cancers of the breast, colon, and gastrointestinal tract and in neuroblastoma. However, he suggested that, while telomere length may not be a good indicator of early neoplasia, it might be a useful predictor of prognosis. Ohyashiki suggested that telomere length may be a good diagnostic tool when telomerase activity is not elevated. Shay cautioned the discussants that there is presently no way to accurately measure telomere length and that, while some laboratories measure average telomere length, other laboratories assay for peak activity. Thus, the methodology for measuring telomere length has not been standardized, making it difficult to compare studies across laboratories.

Clinical and Translational Applications

Gazdar noted that considerable research is needed to determine which cancers might be the best targets for early detection.
by telomerase. Focusing on the possibility that telomerase might eventually be useful in screening populations, Tarin suggested that common cancers should be targeted and noted the importance of obtaining samples noninvasively to increase compliance with screening. Sidransky indicated that more studies are needed to establish laboratory gold standards for telomerase against which telomerase in noninvasively obtained samples could be compared.

Shay discussed the need to investigate the utility of combinatorial biomarkers to detect cancer early (e.g., using K-ras mutations in combination with telomerase to detect colon cancer or microsatellite alterations with telomerase to detect head and neck cancer). The panelists agreed on the merits of studying multiple markers in combination.

Workshop Recommendations

In a formal report to the NCI, the panel concluded that telomerase is almost always detected before the onset of local invasion in breast, colon, lung, and prostate cancers and noted that current concern about interlaboratory measurement variability is likely to be resolved by the impending development of commercial assay kits. The panelists emphasized the importance of accounting for background levels of telomerase in assays. They noted that telomerase can be detected in noninvasively acquired samples and suggested that, to maximize the possible public health impact of early detection, future studies should focus on common cancers and cancers in which the enzyme has already demonstrated potential for early detection. The panel concluded that studies to date on telomerase and the early detection of cancer are encouraging and recommended further investigations in the following three areas:

1) Molecular Technology

• Refinement and standardization of telomerase assays.
   Issues of current concern include variability in interlaboratory measurements, the use of enzymatic controls, and the use of internal controls.
• Development of an in situ assay for telomerase and further development of assays to measure hTR and telomere length.
   A standardized method to visualize telomerase RNA in situ, i.e., in fixed tissues, is needed. Improved, standardized methods are needed for measuring telomere lengths, quantitating telomerase RNA levels, and extracting telomerase.
• Establishment of tissue repositories to store frozen samples.
   Paired samples of neoplastic–non-neoplastic adjacent tissues should be frozen and stored. The samples can be used to compare results using current and future assay methods between different laboratories.

2) Basic Biology

• Regulation of telomeres and telomerase expression.
   Many aspects of the basic regulation of telomeres are not understood. More basic biology studies are needed, particularly on regulation of telomere length and telomerase activity.

3) Clinical and Translational Applications

• Tissue- and organ-specific expression of telomerase.
   More information is needed about telomerase expression in normal proliferative tissues and how to account for normal expression in analyses of telomerase in cancers.
• Laboratory validation of telomerase, hTR, and/or combinations with other biomarkers.
   Continued research is needed to validate telomerase as a biomarker for cancers. In addition, the validity of telomerase alone or in combination with other biomarkers, including hTR, should be evaluated.
• Screening validation of telomerase, hTR, and/or combinations as a biomarker for preinvasive neoplasia and early invasive neoplasia in noninvasively obtained samples.
   The focus should be on common cancers in which there may be some prospect for using telomerase as an early detection marker. Most studies to date suggest that telomerase is detected early in breast cancer and lung cancer. Further studies should be performed on these and other cancers.

In addition, the panel recommended further research in areas not related to early detection, i.e., beyond the scope of this workshop, but of potential importance in determining the prognosis and treatment of patients with cancer:

• Identification of cancers in which telomerase activity predicts clinical outcome.
   Evidence to date suggests that telomerase may predict outcome in meningioma, neuroblastoma, acute myeloid leukemia, and breast and gastrointestinal cancers.
• Evaluation of telomerase utility in medical and surgical practice.
   Telomerase may have utility for decisions about the treatment of ordinary meningiomas. In addition, there may be eventual utility for decisions about the treatment of pancreatic and thyroid cancers.
• Evaluation of telomerase as a marker for the presence of minimal residual disease.
   In patients undergoing chemotherapy, telomerase activity might be useful as a marker of minimal residual disease. The presence of telomerase might indicate the failure of chemotherapy.

Future of Telomerase in Early Detection

Data presented at this workshop suggest that telomerase eventually may have utility as a molecular marker for the early detection of carcinogenesis. However, the field is new, the existing data are limited, and more research is needed.

Early detection has two major components, early diagnosis and screening. Most presentations at the workshop focused on how well the presence of telomerase in tissue or organ samples correlated with the histopathology of those samples; i.e., they were preliminary laboratory validations in the area of early diagnosis. Further studies are needed to determine the sensitivity and specificity of telomerase. In addition, similar studies are needed to determine the value of hTR and combinations of biomarkers such as p53 or K-ras with telomerase or hTR. These studies could serve a dual purpose. In the area of early diagnosis, they could provide insights into which manifestation of telomerase might best detect specific cancers and at which stages. In the area of screening, they might form the basis for establishing cancer-specific gold standards to evaluate screening validity, i.e., the sensitivity and specificity of telomerase (or hTR, etc.) in...
samples obtained noninvasively (or minimally invasively) from asymptomatic individuals.

The ultimate goal of early detection is screening. Fig. 1 shows where opportunities for early detection may lie along the continuum of carcinogenesis. Technology permitting, screening can identify individuals with disease at the earliest preclinical stages. Some of the workshop presenters (notably, Tarin) addressed the issue of screening. While telomerase is far from ready for use in screening programs, it may be useful to consider the process that will determine when and if it becomes ready.

The highest priority is that there be a suitable test to use in the program, i.e., one that, in comparison to an accepted gold standard, separates individuals with and without a particular cancer with reasonable sensitivity and specificity (4). Next, the test must be acceptable to the population screened. Screening is practical only when samples can be obtained with relative ease from the population to be screened. The general asymptomatic population might be willing to provide urine and blood samples or oral rinses but not samples that might be obtained with some discomfort, such as bladder washings or fine-needle breast aspirates. On the other hand, individuals at high risk might be willing to tolerate some discomfort if they perceive a possible benefit.

Next, the screening agent must be able to detect carcinogenesis earlier in the neoplastic process than is currently possible using other screening agents. For example, mammography can detect carcinomas in situ. Telomerase might be of value as a screening agent for breast cancer in populations at high risk if its presence in fine-needle aspirates revealed carcinogenesis at an earlier step in the natural history of this cancer or with greater sensitivity and specificity than is otherwise possible. The incidence of and mortality from cancers discussed by the workshop panelists are shown in Table 1, along with methods for obtaining samples discussed by the panelists.

To maximize public health impact, screening should be targeted at cancers having the highest incidence. However, the public health also might benefit by screening individuals at high risk of lower incidence cancers with high mortality. At present, it appears that, of the high-incidence cancers, telomerase might eventually have utility in screening for breast, colon, and lung cancers. Of the lower incidence cancers, telomerase might eventually have utility in screening for bladder cancer, oral cancer, and leukemia.

The issue of whether treatment is available is also important and raises ethical issues about the harm versus benefit of pro-

Table 1. Projected incidence of and mortality in 1997 in the United States from cancers in which telomerase is currently being studied and examples of biologic specimens potentially useful for screening or early diagnosis using telomerase (22)

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Incidence*</th>
<th>Mortality*</th>
<th>Biologic specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder</td>
<td>54 500</td>
<td>11 700</td>
<td>Urine-exfoliated cells, bladder washings</td>
</tr>
<tr>
<td>Brain</td>
<td>17 600</td>
<td>13 200</td>
<td>Cerebrospinal fluid (potential)</td>
</tr>
<tr>
<td>Breast</td>
<td>181 600</td>
<td>44 190</td>
<td>Fine-needle aspirates, nipple aspirates</td>
</tr>
<tr>
<td>Colorectal</td>
<td>131 200</td>
<td>54 900</td>
<td>Colon washings, endoscopic biopsy specimens</td>
</tr>
<tr>
<td>Oral/pharyngeal</td>
<td>30 750</td>
<td>8440</td>
<td>Oral rinses</td>
</tr>
<tr>
<td>Leukemia</td>
<td>28 300</td>
<td>21 310</td>
<td>Blood, bone marrow</td>
</tr>
<tr>
<td>Lung</td>
<td>178 100</td>
<td>160 400</td>
<td>Bronchoscopic biopsy specimens, sputum</td>
</tr>
<tr>
<td>Prostate</td>
<td>334 500</td>
<td>41 800</td>
<td>Biopsy specimens, prostate massage, semen</td>
</tr>
<tr>
<td>Pancreatic†</td>
<td>27 600</td>
<td>28 100</td>
<td>Pancreatic duct brushings</td>
</tr>
<tr>
<td>Thyroid</td>
<td>16 100</td>
<td>1230</td>
<td>Fine-needle aspirates</td>
</tr>
</tbody>
</table>

*Excludes in situ carcinomas except for bladder.
†The difference between incidence and mortality reflects declining incidence with residual mortality, high lethality, and overreporting on death certificates.

Fig. 1. Opportunities for early detection of cancer and for intervention.
viding to an individual information that cannot be acted upon. This is particularly important for cancers in which telomerase might be able to identify preinvasive neoplasia. Preinvasive neoplasia does not always lead to invasive neoplasia. No solution to this problem can be offered at present, but it should be considered as the research on telomerase progresses. Other factors are also important in screening programs, i.e., cost versus benefit and the need to make screening available on a continuing basis. But telomerase research is far from the point at which these areas need immediate consideration.

Telomerase might have potential in the control of cancer through early detection. Whether its potential will be realized will be determined by continuing research.

References


Notes


J. W. Shay is on the Scientific Advisory Board of Geron Corporation (Menlo Park, CA) that conducts research on telomerase and age-related products.

We thank Drs. Douglas Weed, Ernest Hawk, and Susan Rossi for their comments and suggestions.

Manuscript received October 24, 1996; revised February 18, 1997; accepted February 26, 1997.