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Clinical Implications of Telomerase in Cancer

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Cellular immortality is believed to be a critical step in tumorigenesis. As an important component of the telomere maintenance mechanism, the activation of the enzyme telomerase is tightly associated with cellular immortality and cancer. Telomerase expression is detected in a majority of tumours, but is absent in most somatic tissues and correlates to clinical outcome in a number of cancer types. Telomerase expression is associated with the stage of differentiation but not necessarily with the rate of cell proliferation. Data also indicate that inhibition or absence of telomerase may result in cell crisis in cancer cells and tumour regression in cancer patients. These results suggest that cancer therapy based on telomerase inhibition could be a more effective and safer treatment for cancer, as well as provide a more accurate means for diagnosing and predicting clinical outcome in cancer. Complete understanding of the role of telomerase in tumorigenesis through well-designed clinical studies will have a significant clinical impact on the treatment and diagnosis of cancer.

Key words: telomerase, telomeres, cancer therapy, diagnosis, prognosis

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INTRODUCTION

CANCER IS a disease characterised by uncontrolled proliferation of a certain cell population in the body, a process that eventually affects normal bodily functions. Previous attempts to treat cancer have focused on understanding mechanisms that control cell growth and proliferation. These studies resulted in the discovery of numerous oncogenes and tumour suppressor genes that are involved in tumorigenesis [1]. However, as more genes involved in cancer are discovered, it is becoming clear that the control of cellular growth is ripe with redundant mechanisms that could be exploited by a cancer cell to its selective advantage. Previously overlooked, another characteristic of cancer that is gaining more attention is the unlimited replicative capacity or immortal nature of malignant cells [2].

In some respects, the limited replicative potential of somatic cells, dictated by telomere shortening due to the end-replication problem, can be thought of as the ultimate tumour suppressor mechanism [3]. By limiting the number of divisions a cell can undergo, eukaryotic organisms possess an inherent genetic mechanism to control for unchecked cellular proliferation. Unfortunately, cancer cells have found a way to overcome even this obstacle. However, the good news is that there may be only one major pathway: the activation of the enzyme telomerase by cancer cells, to achieve cellular immortality [4–7]. This hypothesis suggests that, compared to the mechanisms that control cell growth, there may be less redundancy in the mechanisms that are involved in the acquisition of unlimited replicative potential. Thus, therapeutic and diagnostic approaches targeted to the immortal nature of malignant cells may provide more universal and effective means for diagnosing and treating cancer.

TELOMERASE AS A THERAPEUTIC TARGET FOR CANCER

Since the original work by McClintock in 1941 [8], it has been known that telomere structures are necessary for maintaining chromosomal integrity. In vertebrate organisms, telomerase is the main mechanism that is presently known that can stabilise the loss of telomeres (for possible alternative mechanisms, see the article in this Special Issue by T.M. Bryan and R.R. Reddel, pages 767–773). Thus, a rationale for exploiting telomerase as a therapeutic target for cancer is that inhibition of telomerase will cause critical telomere shortening that will lead to chromosomal instability and cell death. Most somatic cells, regardless of their rate of proliferation, undergo a steady rate of telomere loss and do not possess detectable telomerase activity [7]. Furthermore, telomerase expression is closely associated with cellular immortality and the early stage of differentiation, but not with the rate of proliferation *per se* [9, 10]. In other words, whereas quiescent immortal cells downregulate telomerase expression suggesting that cellular division is necessary for the activation of telomerase, rapidly dividing well-differentiated cells do not express any detectable level of telomerase activity even when tested with the highly sensitive TRAP (Telomeric Repeat Amplification Protocol) assay (see the

article in this Special Issue by Holt and colleagues, pages 761–766). This evidence suggests that telomerase inhibition can provide more selective treatment for malignant cells than the traditional cancer therapeutics that act indiscriminately on any proliferating cells.

A large body of data now exists to support the hypothesis that the activation of telomerase may be a critical mechanism for tumour progression. Since the development of the sensitive TRAP assay for measuring telomerase activity, telomerase expression in numerous malignant, premalignant, and normal tissues has been tested [7, 11]. Data from these studies show that telomerase activity is present in almost all tumour-derived cell lines and the majority of malignant tumours but not in most somatic tissues. Inhibition of telomerase activity using an antisense transcript of human telomerase RNA (hTR) induces telomere shortening and crisis in immortal cells [12]. Stage IV-S neuroblastoma patients with telomerase-negative tumours undergo spontaneous tumour regression, whereas patients with telomerase-positive tumours have poorer clinical outcome [13]. Furthermore, telomerase-negative cell populations spontaneously arising from telomerase-positive parental cells undergo telomere shortening and crisis in culture (R.R. Reddel, Children's Medical Research Institute, New South Wales, Australia). Although most of these data provide only indirect support for the importance of telomerase in cancer, they demonstrate that telomerase is a novel and important target for cancer drug discovery.

A single somatic cell with maximum replicative potential of approximately 50 doublings can theoretically generate a biomass of 2^{50} cells, or >1000 kg (10^9 cells/g). Thus, there is a question as to the necessity of telomere stabilisation in tumour progression. Harley and associates point out that these calculations overlooked the multiple mutations that are required for tumorigenesis, and the small fraction of proliferating cells present in most tumours [14]. When the 'multi-hit' nature, the low growth fraction, necrosis, and apoptosis are taken into account, it is estimated that a cancer cell must undergo more than 80 doublings before it generates a biomass of appreciable size. Therefore, activation of telomerase to compensate for telomere loss and maintain chromosomal integrity is believed to be an important factor in the progression of most tumours.

Since it may take several cell divisions for the telomeres of cancer cells to reach a critical length, a therapeutic based on the inhibition of telomerase need not be immediately cytotoxic to telomerase-positive malignant cells. On average, cancer cells possess shorter telomeres than normal cells [13, 15, 16]. Thus, without telomerase to maintain telomere length in dividing cells, cancer cells should approach critically short telomere length faster than normal cells. This factor further suggests that the potential side-effects of an inhibitor for normal telomerase-positive cells in the body will be minimised.

Cellular immortality in germ-line cells is one of the most important criteria for the propagation of species. Therefore, it is not surprising that the germ-line cells possess telomerase activity [7]. Given the telomerase expression in germ-line cells, one possible side-effect of a telomerase inhibitor could be an effect on the reproductive system. If there was an effect, then one could envisage that sperm or ova from a patient could be collected and stored prior to the therapy for future reproductive purposes. Certain populations of stem cells, T- and B-lymphocytes, proliferative regions of hair follicle and inflammatory lesions of the skin and liver, have been also shown to be weakly positive for telomerase activity by the TRAP assay [10, 17–22]. These cells must undergo a higher than average number of cell divisions either to regenerate cells with rapid turnovers or in response to an antigenic challenge. Thus, activation of telomerase may be required for these cells to prevent higher than average telomere loss caused by a high number of cell divisions. Although these cells possess telomerase activity (albeit relatively weak), there is still a reduction of telomeres *in vitro* or *in vivo* in all the systems studied [10, 17, 23–26]. The reasons for this observation are not yet clear. It may be that the level of telomerase activity in these cells is not sufficient to compensate for the loss of telomeric repeats, or that only a very small subpopulation of the cells are positive to telomerase. Furthermore, most of the stem cells have relatively long telomeres, and are in quiescent G_0 stage when telomerase is not activated. These observations suggest that telomerase inhibition therapy on these telomerase-positive stem cells and blood cells may not have a significant negative impact. However, the presence of telomerase expression in these cell types indicates some potential for side-effects during telomerase inhibition therapy and should be examined in animal models.

TELOMERASE AS A DIAGNOSTIC MARKER OF CANCER

The recent development of the sensitive TRAP assay to detect and measure the presence and the level of telomerase activity from small tissue samples has allowed the evaluation of telomerase expression in a wide variety of cancer specimens [7]. At present, numerous data show specific expression of telomerase in the vast majority of tumours tested to date (Table 1). These results are consistent with telomerase being the most widely expressed and specific cancer marker presently known. According to a recent review [11], 758 of 895 (85%) of malignant tumours, but none of 70 normal somatic tissues, expressed telomerase activity. This strong association of telomerase activity with malignant tissue is good evidence that telomerase can be an important marker for diagnosing cancer. As mentioned above, all germ-line tissues tested (4/4) expressed telomerase activity, and weak telomerase activity is also detectable in peripheral blood leucocytes (PBL) and in certain stem cell populations. Although the results from telomerase-negative normal tissues show that normal amounts of blood material do not contribute to significant background problems, telomerase activity from normal PBLs may give rise to a telomerase signal, especially for tumour samples that have high numbers of infiltrating lymphocytes [20, 21]. Some (19/310, 6%) histologically normal tissues adjacent to tumours are also telomerase-positive. Since the TRAP assay is sensitive enough to detect telomerase activity from as little as 1–10 telomerase-expressing cells, telomerase activity from phenotypically normal tissues adjacent to the tumour may be originating from previously undetected infiltrating cancer cells. This result demonstrates that telomerase detection by the TRAP assay may be a more sensitive means for detecting infiltrating cancer cells in tumour margins and may aid in more effective surgical procedures. Lastly, 38 of 266 (14%) benign and premalignant tissues have also been found to express telomerase activity. Telomerase activity from these

*Table 1. Telomerase activity in malignant and normal tissues**

Malignant (No. positive/No. tested)	Normal (No. positive/No. tested)	Adjacent to tumour (No. positive/No. tested)	Benign and premalignant (No. positive/No. tested)
Neuroblastoma (99/105)	Germ-line (4/4)	Head and neck tumour (6/16)	Prostate PIN3 (3/5)
Colon (53/56)	Somatic tissues (0/70)	Wilm's tumour (2/6)	Gastric adenoma (1/2)
Skin (56/61)		Prostate cancer (3/25)	Breast fibroadenoma (2/3)
Breast (117/125)	PBLs 10^3 cells (0/124)	Breast cancer (3/42)	Hepatitis (19/38)
Uterine (18/20)	PBLs 10^4 cells (55/124)	Lung cancer (3/68)	Cirrhosis (6/8)
Ovarian (7/7)	PBLs 10^5 cells (100/124)	Gastric cancer (2/85)	BPH (1/20)
Lung (120/147)		Renal cancer (0/55)	Intestinal metaplasia (1/13)
Prostate (27/31)		Neuroblastoma (0/13)	Anaplastic astrocytoma (1/16)
Gastric (72/85)			Benign meningioma (4/27)
Hepatic (28/33)			Colon polyps (0/20)
Brain (43/59)			Colon adenoma (0/1)
Renal (40/55)			Ganglioneuroma (0/4)
Haematological (78/111)			Leiomyoma (0/103)
			Miscellaneous breast disorders (0/6)
Total (758/895), 85% positive		Total (19/310), 6% positive	Total (38/266), 14% positive

*Compiled from review article by Shay and Wright (1996) [11].
PBLs, peripheral blood leucocytes.

types of specimens may be due to the presence of previously undetected malignant cells. If telomerase activity in these samples is derived from histologically benign cells that have undergone malignant transformation, then it indicates that telomerase expression can be utilised to diagnose cancer at an early stage, and to predict the outcome of disease.

With the results shown in Table 1, a rough estimation of the sensitivity and specificity of telomerase can be determined. For a conservative estimate, telomerase-positive samples from tissues adjacent to tumour and benign/premalignant tissues are treated as false-positives. Combined results of telomerase expression from 895 malignant and 646 non-malignant (70 normal somatic, 310 tissues adjacent to tumour, and 266 benign and premalignant tissues) indicated specificity of 91%, sensitivity of 85%, positive predictive value of 93%, and negative predictive value of 81%. These preliminary results demonstrate that telomerase has a promising potential as a useful diagnostic marker for cancer.

Although telomerase seems to be a very specific marker for cancer, establishing the clinical utility of this enzyme as a tumour marker depends on additional criteria. Highly useful diagnostics should fulfill current unmet needs in traditional cancer diagnosis. In general, the current gold standard for most cancer diagnoses is histopathology, which in most cases provides an accurate assessment of the suspected tissue biopsies. Therefore, the most significant clinical utility of telomerase could be in the diagnosis of cancer through easily obtainable bodily fluids such as urine, blood, sputum, and materials derived from various washes. For example, traditional cytological diagnosis of bladder cancer is not very accurate, making this a cancer for which more sensitive diagnostic markers could make a significant impact. Telomerase activity has been detected in cells collected through bladder washes and voided urine of patients with bladder cancer, indicating that telomerase may be an important marker for the diagnosis, monitoring and screening of bladder cancer (D. Tarin, University of Oxford, Oxford, U.K.; M. Muller, Universitätsklinikum Benjamin Franklin, Germany; G. Dalbagni, Memorial Sloan-Kettering, U.S.A.). Furthermore, diagnosis of lung and head and neck cancer through testing of patient's sputum, and of metastatic cancers, such as breast or prostate cancer, through testing of patient's blood are some of the other potential diagnostic applications of telomerase. In some cancer types, where traditional histopathology or cytology, especially in the cases of the fine needle aspirates (FNA) and PAP (cervical) smear, result in a high proportion of indeterminant cases, more sensitive and specific markers, such as telomerase, may be useful in identifying the presence of malignant cancer cells. Lastly, a simple and reliable method of detection that is easy to use is another important component of a successful diagnostic marker. This condition appeared to be relatively difficult to achieve for a low-copy marker such as telomerase, but significant progress has been and continues to be made in this area as discussed in the following sections.

TELOMERASE AS A PROGNOSTIC MARKER OF CANCER

There are several studies that demonstrate telomerase expression correlation with clinical outcome in certain cancers. The presence of telomerase activity has been shown to correlate with poor clinical outcome in gastric cancer [27]. High levels of the enzyme correlate with poor clinical outcome in neuroblastoma, whereas patients with metastatic IV-S neuroblastoma without telomerase activity experience spontaneous regression of the tumours [13]. Clinical outcome and several prognostic indicators of breast cancer have been shown to have a statistically significant correlation with the level of telomerase activity (N. Kim, Geron Corporation, U.S.A. and G. Clark, University of Texas, San Antonio, U.S.A.). Lastly, a high level of human telomerase RNA (hTR) has been shown to have a statistically significant correlation with poor clinical outcome on 159 neuroblastoma patients (C.P. Reynolds, Children's Hospital of Los Angeles, California, U.S.A.). These data demonstrate that telomerase can be a useful marker for predicting outcome of disease and can provide new information for determining appropriate treatment for a cancer patient. The value of a novel prognostic marker will be most evident in a

cancer type where there are clearly defined treatment options that are dependent upon the aggressiveness of the tumour in question. Thus, a telomerase-based prognostic could be useful in accurately identifying a population within node-negative breast cancer patients, as well as other cancers, that are likely to experience tumour recurrence and will benefit from more aggressive adjuvant therapy. Furthermore, telomerase may be used to determine accurately a subgroup of IV-S neuroblastoma patients that is likely to undergo spontaneous remission of tumours, which will prevent unnecessary toxic therapy being given to these young individuals.

Definitive studies to provide further assessment of the prognostic utility of telomerase will be an important next step in telomerase research. Well characterised retrospective tumour banks with complete clinical information, including disease outcome, could be used for determining the prognostic utility of telomerase. However, in dealing with retrospective specimens, it is also important to have a comprehensive record of how tissues were prepared and stored. Because telomerase holoenzyme is sensitive to heat, RNase, and proteases, the TRAP assay is mainly performed on frozen specimens, including cryosections, where enzymatic activity of telomerase is maintained. For this reason, a prospective study where appropriate sample collection procedures and storage conditions could be instituted from the beginning has the advantage over testing retrospective specimens. Incorporating methods to determine sample integrity is an important criterion for a valid telomerase study, and because the TRAP assay detects enzymatic activity of telomerase, it is important to determine the general enzymatic conditions of the tissue specimens. Currently, there is no easy-to-use optimal enzymatic assay that is proven to be highly reflective of telomerase stability. Alkaline phosphatase is a relatively stable enzyme, but its relative ease of use allows a simple means of determining a rough estimate of the enzymatic integrity of a test extract [28]. In addition to testing extracts for presence of a house-keeping enzyme, such as alkaline phosphatase, measurement of the level of house-keeping RNA, such as the 28S ribosomal RNA, can also be used to test sample integrity. Present data indicate that the prognostic utility of telomerase may be fully realised by measuring the level of telomerase expression. Thus, a telomerase assay for use in a prognostic study should preferably be at least semiquantitative. Furthermore, the level of telomerase expression may be more useful if standardised to cellularity. Recent data indicate that the level of telomerase activity in a biopsied specimen is directly proportional to the percentage of cancer cells in the tumour (N. Kim, Geron Corporation, California, U.S.A.). Thus, the amount of telomerase in a tumour can be described by standardising to the percentage of cancer cells in the test sample determined by histological evaluation, or standardised through cellular markers such as RNA content.

In addition to the clinical utilities in cancer diagnosis and prognosis, telomerase expression could be used to monitor the effectiveness of cancer therapy. As discussed in the previous section, telomerase expression correlates with cellular immortality and the stage of differentiation. Thus, detection of telomerase expression can be used to monitor the state of cellular differentiation during traditional cancer therapy such as the retinoid treatment for acute promyelocytic leukaemia (APL) [29]. Because telomerase is a specific marker of cancer, the level of telomerase expression could be used to determine the number of cancer cells present in a patient undergoing treatment, and could also be used to detect the presence of cancer cells in a patient's bone marrow used in autologous bone marrow transplantation. Such information may aid in determining the most effective therapy regimen for the patient and help reduce the number of cancer recurrences after transplantation due to occult cancer cells present in the bone marrow.

METHODS FOR DETECTING TELOMERASE

There are currently two published targets for detecting telomerase expression: the biochemical activity of telomerase; and the RNA component of telomerase. A biochemical primer-extension-based assay for human telomerase was developed in 1989 [30] based on the ciliate telomerase assay [31]. However, because of the rarity of telomerase in cells, estimated to be approximately 200 copies per cell, broad evaluation of telomerase expression in primary tumour biopsies was not practical until the development of the sensitive TRAP assay. In the TRAP assay, the biochemical properties of telomerase (i.e. its binding to and extending of an oligonucleotide substrate with *de novo* telomeric repeats) are combined with a nucleic acid amplification method such as the polymerase chain reaction (PCR). The current TRAP assay based on PCR amplification is a one-tube assay that is able to detect telomerase activity from a single cell. One active molecule of the telomerase holoenzyme could produce many extension products which are subsequently detected in the TRAP assay. The enzymatic activity of telomerase provides additional target amplification, which makes the TRAP assay theoretically the most sensitive assay for detecting telomerase expression. Currently, most TRAP applications utilise gel electrophoresis to visualise the TRAP products, and require about 5–7 h to obtain a result. Recently, improved primer designs and PCR internal control have been used to measure quantitatively the level of telomerase activity by the TRAP assay (N.W. Kim and F. Wu, Geron Corporation, U.S.A.). The TRAP assay can utilise amplification systems other than PCR (e.g. LCR, bDNA, NASBA, etc.) to improve ease of use and quantitation. Future assay systems will be more user-friendly and quantitative and will provide valuable tools for evaluating and utilising telomerase as a diagnostic and prognostic marker for cancer.

Although the TRAP assay provides the most biologically relevant information, that is the biochemical activity of telomerase, and is theoretically the most sensitive method for detecting telomerase expression, there are some instances where the TRAP assay may not be preferred for a routine evaluation of telomerase expression. Because the assay detects biochemical activity of telomerase, which is sensitive to heat, RNase and protease contamination, any fixed or heated clinical specimens are not the optimal specimens for TRAP analysis. Cloning of the RNA component of human telomerase provides another means of measuring telomerase expression in clinical specimens [12]. Standard Northern analysis and RT-PCR assay were previously used to detect and measure telomerase RNA expression [12, 32, 33]. An assay that measures hTR levels can be used on fixed or heated specimens in addition to frozen samples. Numerous sensitive assays for measuring RNA (e.g. S1 nuclease digestion, RT-PCR, bDNA, NASBA, etc.) are currently available

and can be applied to hTR detection. Furthermore, hTR detection can be used to detect telomerase-positive cancer cells on histological sections using *in situ* hybridisation (N.W. Kim, Geron Corporation, California, U.S.A.). Unlike the biochemical activity of telomerase, hTR is expressed at low levels even in normal tissues that are negative for telomerase activity. Current data indicate that there is approximately a 2 to 20-fold induction of the hTR level, depending on the assay used and type of tissue, in cancer tissues that are positive for telomerase activity compared with normal tissues that lack detectable levels of the enzymatic activity [12, 17, 32, 33]. This difference in the level of hTR between cancer and normal cells is sufficient to identify telomerase-positive cancer cells from a background of telomerase-negative normal cells by the *in situ* hybridisation assay. However, the background level of hTR may cause difficulty in determining telomerase expression, using tube-based assays such as Northern or RT-PCR analysis, from a small number of cancer cells in a heterogeneous tumour. Thus, quantitation of the hTR level and its standardisation to cellularity may be important considerations for utilising fully hTR as a diagnostic and prognostic marker of cancer.

Development of sensitive and reliable technologies and assays will continue to be an important driving force behind the development of telomerase as a novel diagnostic marker for cancer. Further clinical studies will determine which telomerase assay format, TRAP or hTR detection, will be most appropriate for each cancer type. Cloning of the protein component of telomerase will provide additional assays for telomerase detection such as ELISA, immunohistochemistry and mRNA detection. These assays could be used to improve sensitivity, specificity, reliability and ease of use for detecting and measuring the level of telomerase expression.

TELOMERE AS A DIAGNOSTIC MARKER AND A THERAPEUTIC TARGET

Since the major function of telomerase in cells is the maintenance of telomere length, there is a relationship between telomere dynamics and telomerase expression. Thus, telomeres could be an alternative therapeutic target for cancer, and the clinical utilities of telomere length measurement should be investigated further in diagnostic applications in cancer as well as non-cancerous diseases. As an alternative to telomerase inhibition therapy, selectively destabilising or 'poisoning' telomeres of cancer cells may provide another means of treating cancer. A small molecule, compound or nucleoside analogue that integrates specifically at telomeres with or without the aid of telomerase, and destabilises the structure of telomeres, may be one such therapeutic drug.

Telomere length measurement will provide important information in determining the maximum replicative capacity of cancer cells that are being subjected to telomerase inhibitor therapy. This parameter will determine how long telomerase inhibitor therapy has to be given, and whether additional cytoreductive therapies will be required. Thus, the measurement of telomere length will be an important means for monitoring the effectiveness of telomerase inhibition therapy in patients. As mentioned in the previous section, the average telomere length of cancer cells is generally shorter that that of their normal counterparts. Thus, a cancer diagnostic assay based on the measurement of telomere length, especially in an *in situ* hybridisation format, should be feasible and helpful.

Diagnostic tests based on telomere length measurement also have application in diseases other than cancer. Telomere lengths in vascular tissues with high cellular turnover due to haemodynamic stress (more susceptible to atherosclerosis) are shorter than in vascular tissues with less cellular turnover [34]. Telomere lengths of a subpopulation of T-cells from patients with AIDS were also recently shown to be shorter than telomere lengths of T-cells from a control group [35]. These data demonstrate that a diagnostic and prognostic test based on telomere length measurement for cardiovascular diseases and HIV-infected patients may be of benefit. Lastly, there is a gradual decrease in telomere lengths of cells from fetal tissue, to cord blood, to adult bone marrow [24], demonstrating that the difference in length between fetal and maternal cells could also be used to identify fetal cells circulating in maternal blood which could be used for non- invasive tests to detect genetic abnormalities in the fetus.

CONCLUSION

Telomerase continues to be a highly selective and potentially universally effective target for cancer therapy. Unlike the traditional cancer therapeutic that mainly acts on rapidly proliferating cells, telomerase inhibition cancer therapy could provide an additional level of specificity for cancer cells, because telomerase is shown to be associated mainly with cellular immortality and the stage of differentiation. It is becoming clear, however, that the expression of telomerase is not solely limited to germ-line and cancer cells. Expression of telomerase in stem cells and haematopoietic cells suggests that the toxicology profile of a telomerase inhibitor should be evaluated in an animal model. Specific association of telomerase with cancer cells continues to be validated in more cancer types. The present data suggest that telomerase may be the most specific and universal cancer marker known to date. Furthermore, a number of studies have shown that telomerase expression correlates with clinical outcome in some cancers. This promising diagnostic and prognostic potential of telomerase should be further studied in well-designed and executed clinical studies to determine definitively the full clinical utility of telomerase-based cancer diagnostics. Further improvements in the telomerase detection methodologies, with respect to ease of use, will aid in this effort and will make the technologies more accessible to clinicians and patients.

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