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Telomerase detection in the diagnosis and prognosis of cancer

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Abstract

Telomerase, a critical enzyme responsible 'for cellular immortality, is usually repressed in somatic cells except for lymphocytes and self-renewal cells, but is activated in approximately 85% of human cancer tissues. The human telomerase reverse transcriptase (hTERT) is the catalytic component of human telomerase. In cancers in which telomerase activation occurs at the early stages of the disease, telomerase activity and hTERT expression are useful markers for the detection of cancer cells. In other cancers in which telomerase becomes upregulated upon tumor progression, they are useful as prognostic indicators. However, careful attention should be paid to false–negative results caused by the instability of telomerase and of the *hTERT* mRNA and the presence of PCR inhibitors, as well as to false–positive results caused by the presence of alternatively spliced *hTERT* mRNA and normal cells with telomerase activity. Recently, methods for the *in situ* detection of the *hTERT* mRNA and protein have been developed. These methods should facilitate the unequivocal detection of cancer cells, even in tissues containing a background of normal telomerase-positive cells.

Abbreviations: BAL – bronchoalveolar lavage; FNA – fine needle aspiration; IHC – immunohistochemistry; ISH – in situ hybridization; hTERT – human telomerase reverse transcriptase; hTR – human telomerase RNA; TRAP – telomeric repeat amplification protocol.

Introduction

Mammalian telomeres are made of many hundreds to thousands units of the simple DNA repeat TTAGGG. Telomeres form a nucleoprotein that cap and protect the ends of linear chromosomes. Because the DNA replication machinery cannot fully replicate the ends of linear DNA molecules, telomeres progressively shorten with each cell division (Watson 1972). Eventually, when a critically short telomere length is reached, cells stop dividing and senesce (Greider 1990; Wright and Shay 1992). This phenomenon is thought to function as a 'mitotic clock' that limits the lifespan of individual cells. Telomerase is a specialized reverse transcriptase that synthesizes telomeric

repeats onto chromosomal ends and thus compensates for the progressive shortening of the telomeres caused by the end-replication problem (Lingner et al. 1995). This enzyme lacks from most somatic human cells and is typically restricted to certain specialized cell types, such as germ cells and stem/progenitor cells of self-renewal tissues, which must perform unusually large numbers of cell divisions (Hiyama et al. 1995c; Wright et al. 1996; Sakabe et al. 1998).

Cancer is a disease characterized by uncontrolled proliferation and invasion into surrounding tissues or distant organs. Most somatic human cells lack telomerase activity, have a limited life span, and require the activation of telomerase for unlimited proliferation. Although telomerase activation is not always concomitant with carcinogenesis, its presence in 85% of more than 3000 tumor samples tested makes telomerase activity the most universal maker of human cancers (Kim 1997; Shay and Gazdar 1997; Dhaene et al. 2000). Recent reports revealed that telomerase activity is upregulated during mouse tumorigenesis in spite of the fact that mice have very long telomeres (Blasco et al. 1996; Broccoli et al. 1996). This observation and others have suggested that telomerase may promote tumorigenesis independently of telomere length. By stabilizing telomeres and supporting the indefinite growth of most cancer cells, telomerase most certainly plays a crucial role in the progression and maintenance of tumors.

An important question, is when telomerase is activated during the multi-step process of carcinogenesis. In some instances, telomerase may already be ubiquitously expressed at the preneoplastic or *in situ* stage; while in other instances, the enzyme may be activated gradually with cancer progression (Shay and Bacchetti 1997). These differences are crucial in dictating whether telomerase might be clinically useful for either diagnostic or prognostic purposes. Although most somatic human cells lack telomerase activity, some tissues contain specialized cells, including germ cells, lymphocytes, stem cells, or certain epithelial cells, that display weak levels of telomerase activity, which can be upregulated concomitantly with growth signals. In such tissues, *in situ* immunohistochemical detection of telomerase may be necessary to determine whether telomerase expression is derived from normal telomerase-positive cells or from cancer cells. Taking these key points into account, telomerase is now being explored as a novel marker for early detection and/or the grading of malignant tumors. The present article reviews the use of human telomerase as a cancer diagnostic marker and as a prognostic tool for predicting the outcome of individual patients.

Detecting human telomerase in clinical materials

Telomerase can be measured by a PCR-based assay called telomeric repeat amplification protocol (TRAP) (Kim et al. 1994). The assay is quite sensitive and can detect as few as 10 telomerase positive cells (Wright et al. 1995). With this high sensitivity, telomerase activity can also be detected in certain normal somatic tissues, especially in proliferative and/or stem cells of self-renewing tissues (such as intestinal epithelium) and activated lymphocytes (Hiyama et al. 1995c, 1996b; Wright et al. 1996). Moreover, this activity is also detectable, albeit at low levels, in some benign tumors such as fibroadenomas of the breast (Hiyama et al. 1996a), hyperplastic nodule/adenomas of the thyroid (Matthews et al. 2001), and colon adenomas (Hiyama et al. 1996b). As a general rule, telomerase activity in normal somatic cells tends to be much lower in comparison to that detected in cancer cells. In clinical samples from tissues containing normal telomerase-positive cells, evidence of cancer cells requires levels of telomerase activity that are significantly higher than those of matched control tissues (Shay and Bacchetti 1997). As the TRAP assay is based on semi-quantitative PCR, a more precise method of quantification might be needed for such samples. To overcome this limitation, a real-time PCR assay (RTQ-TRAP) has been developed that allows quantitative measurements of telomerase activity in tissue samples (Hou et al. 2001). To avoid false–positive results due to contamination of cell samples with lymphocytes, we recommended using a thousand cell equivalents of cell lysate per assay, as proteins extracted from a thousand adult lymphocytes do not produce detectable telomerase activity (Hiyama et al. 1995c; Iwao et al. 1997). To avoid false–negative results, careful attention should be paid to the stability of telomerase and the presence of PCR inhibitors when examining clinical specimens.

Human telomerase activity is associated with the expression of two major components: human

telomerase RNA (*hTR*) (Feng et al. 1995) and human telomerase reverse transcriptase (hTERT) (Nakamura et al. 1997). Recent studies have targeted the expression of these two components as surrogates for telomerase activity and discussed their value as tumor markers. Since hTR is expressed at low levels in all cells, including cells that lack telomerase activity (Koyanagi et al. 2000), detection of the *hTERT* mRNA is believed to be a more reliable marker of the presence of cancer cells in clinical samples. However, the existence of splicing variants of the *hTERT* mRNA that fail to produce telomerase activity (Ulaner et al. 1998) can also be problematic for the use of the *hTERT* mRNA as surrogate for telomerase activity.

Telomerase as a diagnostic marker

Recently, there has been an increasing amount of experimental data on the detection of telomerase activity and/or hTERT expression in clinical materials as a diagnostic tool for various cancers (Table 1).

Head and neck tumors

Most cancer tissues in head and neck lesions show high levels of telomerase expression. Although the viability of cancer cells in these specimens is not particularly high, telomerase activity is often detected in oral washings of patients with oral malignancy (Califano et al. 1996; Sumida et al. 1998). In such specimens, it is difficult to avoid contamination by substances that interfere with PCR, such as necrotic tissue, leukocytes, erythrocytes, dental plaque, and bacteria. The presence of these substances in cancer samples can lead to false-negative results. Although currently limited in its sensitivity, the detection of telomerase activity or *hTERT* mRNA in oral washings is a novel marker indicating the presence of cancer cells shed from the upper aerodigestive tract.

In tumor biopsies, telomerase activity and the *hTERT* mRNA are almost always detected at high levels. Since low levels of telomerase activity are detected in the normal buccal epithelia and in approximately 20% of non-cancerous biopsy samples, a quantitative TRAP assay may be required for cancer diagnosis.

Thyroid and breast tumors

Because thyroid and breast lesions are easily palpable, fine needle aspiration (FNA) is widely used as a diagnostic tool for cancer detection in these lesions. For tumors of the thyroid gland, differential diagnosis between follicular adenoma and adenocarcinoma is difficult by FNA cytology alone. The detection of telomerase activity and/or *hTERT* mRNA has been found to be a useful tool for this differential diagnosis; as cancers gave positive signals while adenomas were negative for telomerase expression (Umbricht et al. 1997; Zeiger et al. 1999). However, thyroid tissues often contain lymphocytes, so that telomerase activity and *hTERT* mRNA derived from these inflammatory cells may also be detectable in certain benign diseases, such as Hashimoto thyroiditis (Haugen et al. 1997).

In the breast, normal mammary tissue lacks detectable telomerase activity, while the activity is expressed in 80–90% of ductal carcinoma *in situ* (DCIS) lesions and 90% of invasive breast cancers (Hiyama et al. 1996a; Umbricht et al. 1999). *hTERT* mRNA is detected at high frequency in breast cancers, where its levels are relatively high (Bieche et al. 2000). One of the most common problems in using telomerase for breast cancer diagnosis is the presence of telomerase activity in benign fibroadenomas. Approximately 40% of fibroadenoma tissues display low-level telomerase activity (Hiyama et al. 1996a; Pearson et al. 1998). In combination with cytology and with careful attention to benign diseases, the screening of FNA samples for telomerase expression is likely to become a powerful tool for the detection of breast and thyroid cancers (Pearson et al. 1998; Mokbel et al. 1999; Poremba et al. 1999a; Hiyama et al. 2000).

Lung and mediastinum

Sputum, bronchoalveolar lavage (BAL), bronchial brushing, and bronchial washing samples have all been tested in the TRAP assay for the detection of lung cancer cells (Sen et al. 2001). Use of the TRAP assay on sputum samples might hold potential for the early and non-invasive diagnosis of lung cancer. However, since sputum contains an abundance of mucus, which interferes with PCR

Table 1. Telomerase/*hTERT* mRNA as a diagnostic marker.

Organs/samples	Telomerase activity		*hTERT* mRNA	
	Cancer positive (%)	Non-cancerous positive (%)	Cancer positive (%)	Non-cancerous positive (%)
Head & neck				
Oral/Washing	110/195 (56)	70/321 (22)	21/26 (81)	9/39 (23)
Oral/Biopsy	25/26 (96)	9/41 (22)	47/58 (81)	11/13 (85)
Thyroid & breast				
Thyroid/FNA	64/96 (67)	23/155 (15)	44/57 (77)	15/52 (29)
Breast/FNA	210/265 (79)	40/355 (11)		
Chest				
Lung/Sputum	15/42 (36)	0/10 (0)		
Lung/Brushing, BAL	123/188 (65)	16/211 (8)		
Lung/Biopsy	86/128 (67)	0/10 (0)		
Mediastinal LN/FNA			10/16 (63)	18/71 (25)
Pleural effusion	175/205 (85)	20/155 (13)	14/15 (93)	6/15 (40)
Digestive organs				
Esophagus/Biopsy	52/54 (96)	33/48 (69)		
Stomach/Biopsy	23/29 (79)	10/28 (36)	88/101 (87)	62/192 (32)
Colon/Washing	20/34 (59)	0/20 (0)		
Colon/Biopsy	110/126 (87)	57/148 (39)	32/32 (100)	17/49 (35)
Liver/Biopsy	53/86 (62)	17/58 (29)	21/23 (91)	17/63 (27)
Biliary duct/Bile	4/37 (11)	0/25 (0)	10/20 (50)	0/14 (0)
Biliary duct/Biopsy	20/26 (77)	0/10 (0)	6/10 (60)	0/6 (0)
Pancreas/Pancreatic juice	59/72 (82)	2/51 (4)	15/17 (88)	2/19 (11)
Pancreas/FNA	18/18 (100)			
Peritoneal Lavage	102/141 (72)	5/117 (4)		
Genitourinary organs				
Bladder/Voiding urine	374/637 (59)	44/488 (9)	159/179 (89)	6/169 (4)
Bladder/Washing urine	229/302 (76)	6/153 (4)	125/168 (74)	19/165 (12)
Bladder/Biopsy	46/54 (85)	30/56 (54)		
Prostate/Voiding urine[a]	21/33 (64)	1/21 (5)		
Prostate/Biopsy	130/166 (78)	19/136 (14)		
Uterus/Cervical scraping	105/273 (38)	37/233 (16)	14/17 (82)	11/44 (25)
Uterus/Biopsy	138/164 (84)	58/158 (37)	83/104 (80)	1/8 (13)
Others				
Skin/Biopsy	130/159 (82)	11/109 (10)		
Blood/Serum	59/95 (62)	0/80 (0)	4/16 (25)	0/23 (0)

These percentages were calculated from the review papers Dhaene et al. (2000), Hiyama and Hiyama (2002, 2003) and Orlando et al. (2001) and recent numerous reports in addition to our unpublished data.
Abbreviations: BAL, bronchoalveolar lavage; FNA, fine needle aspirates.
[a]Voiding urine after massage.

and other enzyme reactions, the sensitivity of the telomerase assay in sputum is unsatisfactory for the detection of cancer (Sen et al. 2001). In brushing or BAL samples, on the other hands, telomerase activity showed a relatively high sensitivity for the detection of lung cancer cells, but more so for squamous cell carcinoma than for adenocarcinoma. However, BAL samples can contain activated lymphocytes, which can give false-positive results in benign diseases. The clonal expansion of lymphocytes, in particular, can produce strong telomerase activity in BAL samples (Haruta et al. 1999), which may instead reflect the aggressiveness of autoimmunity in certain benign diseases (Hiyama et al. 1998).

Several attempts to detect telomerase activity or *hTERT* mRNA have been reported using pleural effusion and mediastinal lymph node aspiration samples (Yang et al. 1998; Braunschweig et al. 2001; Dejmek et al. 2001; Wallace et al. 2003). Because carcinomas from almost any tumor sites can metastasize to the pleura, pleural effusions

may contain cancer cells originating from other organs such as the breast, ovary, or gastrointestinal tract. In malignant pleural effusions diagnosed by either fluid cytology or pleural biopsy, Yang et al. (1998) detected telomerase activity in 91% of cases with a specificity of 94%, indicating that the measurement of telomerase activity is a useful adjunct to cytology for detecting cancer cells. In this study, the only false positives were three samples from patients with tuberculosis. Thus, in pleural effusions as well as BAL samples, the sensitivity of the telomerase assay for detecting cancer cells is relatively high, with most of the false–positive signals being caused by lymphocytes contamination of non-cancerous lesions.

Digestive organs and peritoneum

Cancers of the digestive system are frequently diagnosed by endoscopic examination. In biopsies of the esophagus, telomerase activity was present in almost all esophageal cancers but was also detected in more than half of non-cancerous tissues, where the activity can be found in the normal epithelial basal cells. Similar results were obtained for biopsies of the stomach and colon. The sensitivity of telomerase activity or *hTERT* mRNA expression for detecting cancerous lesions was high, but telomerase was also present, albeit at lower levels, in non-cancerous tissues, where it localizes to the basal cells of crypts (Hiyama et al. 1996b). Hence, a more precise measurement of the level of telomerase activity in biopsy samples of the gut may be necessary for the diagnosis of cancers. Cells derived from colon luminal washing can also be applied to the TRAP assay for cancer diagnosis. Because washing samples rarely contain basal crypt cells, the specificity of the TRAP assay for colon washing samples was remarkable but at the expense of sensitivity, which was found to be relatively low (Yoshida et al. 1997).

In liver biopsies, detection of telomerase activity or *hTERT* mRNA shows promises for cancer diagnosis, albeit low-level expression of these markers was also reported in non-cancerous tissues (Nagao et al. 1999). In pancreatico-biliary cancers, detection of telomerase activity or *hTERT* mRNA in biopsy samples displayed a high sensitivity for cancer diagnosis. With the exception of pancreatic juice, the assay had a sensitivity that was low for excretion and secretion samples, such as bile (Itoi et al. 1999, 2001). In patients with pancreatic ductal adenocarcinomas, the pancreatic juice contains freshly exfoliated ductal cells that carry very high levels of telomerase activity. Because of its high sensitivity and specificity, the detection of telomerase activity and/or *hTERT* mRNA in pancreatic juice has become a promising new application of cancer diagnosis (Hiyama et al. 1997b; Iwao et al. 1997; Suehara et al. 1997; Morales et al. 1998). Moreover, detection of telomerase activity in pancreatic juice was additionally useful for the differential diagnosis of benign and malignant intraductal papillary mucinous tumors (IPMT) of the pancreas, which can be difficult to distinguish preoperatively (Inoue et al. 2001; Uemura et al. 2003).

Because peritoneal dissemination usually occurs in advanced stages of digestive cancers, telomerase activity in peritoneal lavage samples also showed a high specificity for cancer cell detection. Tangkijvanich et al. (1999) measured telomerase activity in nonmalignant and malignancy-related ascites associated with hepatocellular carcinoma and peritoneal carcinomatosis. Both the sensitivity and specificity of the telomerase assay were higher than those of cytology for diagnosis of the malignancy. The incidence of false–positive for telomerase activity was only of 4%, and all of these false positives showed evidence of lymphocytic contamination. Duggan et al. (1998) also found telomerase activity to be more sensitive than cytology in ascitic samples obtained from patients with ovarian cancer.

Genitourinary organs

Among exfoliating materials, voiding urine is easiest to examine. For the detection of bladder cancer using voided urine samples obtained from bladder cancer patients and controls, the TRAP assay showed the highest sensitivity (67%) and specificity (99%) Ramakumar et al. (1999). Since the viability of cells in voided urine samples varies, the sensitivity of telomerase activity for cancer diagnosis was lower than specificity. As an alternative, high sensitivity could be obtained in urine samples by detection of the *hTERT* mRNA by RT-PCR (Ito et al. 1998; Fukui et al. 2001). While telomerase activity and *hTERT* mRNA might both be useful for the detection of cancer cells in

bladder washings, detection of the *hTERT* mRNA may be preferable for the screening of voided urine (Lee et al. 1998; Fukui et al. 2001).

In voided urine samples obtained after prostate massage, the sensitivity of telomerase activity was higher than that of cytologic examination for the detection of prostate cancer (Meid et al. 2001). As a surrogate for unstable telomerase, *hTERT* mRNA was an even more reliable marker. They reported that the addition of EDTA to a final concentration of 20 mmol/l stabilized the RNA for up to 2 h at 4 °C.

As a potential biomarker of cervical dysplasia, telomerase has also been the focus of intense investigations. In cervical cancers, whether telomerase is activated in pre-malignant lesions remains controversial. According to several studies published on cervical biopsies (Wisman et al. 1998; Zheng et al. 2000; Jarboe et al. 2002), telomerase activity is abnormally present in a remarkably high proportion of high-grade squamous intraepithelial lesions (HSILs), indicating that the activation of telomerase is an early event in the malignant progression of cervical lesions. Still, a more complex situation has been suggested by histochemical studies, which revealed that the hTERT protein was present in the lower suprabasal levels of the normal cervical mucosa (Frost et al. 2000). In cancers, the hTERT protein was relocalized to virtually all levels of the lesional epithelia, concomitantly with the aberrant reexpression of telomerase activity. Thus, for the diagnosis of cervical cancers, a more precise measurement of telomerase activity might be needed, which should be confirmed by the histochemical staining of mirror image specimens. Thus, the detection of *in situ* carcinomas and precancerous lesions in cervical biopsies would likely require the application of methodologies for the *in situ* detection of the *hTERT* mRNA or protein, as described later in this review.

Skin

Skin is a surface organ from which biopsy specimens are easily prepared. Investigations of telomerase activity as a marker of skin cancer showed that epidermal basal cells had low levels of telomerase activity; that telomerase was not activated in the vast majority of squamous cell carcinoma; but that most cutaneous malignant melanoma displayed high-levels of telomerase activity (Parris et al. 1999). To elucidate the correlation between carcinogenesis and telomerase activation in the skin, further studies on skin cancers and related lesions are necessary.

Circulating cancer cells

Irrespective of the tumor type, the blood of cancer patients is likely to contain circulating cancer cells that could potentially be detected using the telomerase assay (Gauthier et al. 2001). The detection of these rare cancer cells in whole blood samples would be predicted to be masked by the potential presence of activated lymphocytes expressing high levels of telomerase activity (Hiyama et al. 1995c; Haruta et al. 1999). To detect circulating carcinoma cells using the telomerase assay, immunomagnetic separation can first be used to isolate epithelial cells from peripheral blood mononuclear cells, after which point the harvested cells can be tested for telomerase activity. In one report, the harvested circulating epithelial cells showed telomerase activity in 70–80% of patients with advanced lung, colon, and breast cancers, suggesting that telomerase activity may become a useful clinical marker of circulating epithelial cancer cells (Gauthier et al. 2001).

One of the most routinely collected bodily fluids is blood plasma, which can easily be prepared by centrifugation of whole blood. If tumor cells undergo necrosis and release their contents, some tumor-specific molecules might be present in plasma that could be detected. Although several studies have addressed the detection of tumor-specific mRNA in plasma, this broader topic is beyond the scope of this review. While it is unlikely that intact telomerase might be detected in plasma, a recent report has detected the *hTERT* mRNA and hTR in plasma (Chen et al. 2000).

Hematopoietic malignancies

The presence of endogenous telomerase in normal hematopoietic stem cells and activated lymphocytes is an important confounding factor that can limit the value of the telomerase assay in the detection of hematopoietic malignancies. Blood samples from patients at the early stages of chronic

lymphoid leukemia (CLL) display low-level telomerase activity, which progressively increases over the course of the disease to reach levels that are much higher than those detected in normal blood samples. Moreover, this increase is accompanied by a net decrease in telomere length (Shay et al. 1996). A series of 58 patients with CLL showed that higher telomerase activity and shorter telomeres were associated with an adverse prognosis (Bechter et al. 1998). In chronic myeloid leukemia, telomerase activity is not increased over that of normal peripheral mononuclear cells, but decreases in telomere lengths are observed, which correlate with shorter intervals to the blast crisis phase (Iwama et al. 1997). In acute lymphoblastic leukemia, smaller studies have found telomerase activity to be variable (Shay et al. 1996). With the exceptions of Hodgkin's lymphoma and chronic myeloid leukemia, all of acute myelogenous leukemia, multiple myeloma, plasma cell leukemia and non-Hodgkin's lymphoma exhibited marked increases in telomerase activity that were well above that of normal peripheral mononuclear cells (Norrback et al. 1998; Xu et al. 2001).

In situ detection of the hTERT mRNA and protein

When using RT-PCR or the TRAP assay to detect the *hTERT* mRNA or telomerase activity, the presence of normal telomerase-positive cells, such as lymphocytes or basal epithelial cells, can cause false–positive results. Methodology for the *in situ* detection of telomerase in individual cells would be expected to solve this problem. An *in situ* TRAP assay had previously been developed to detect the telomerase activity, but this methodology could only be used on fresh viable cells (Ohyashiki et al. 1997b). The use of *in situ* hybridization (ISH) to detect components of the telomerase complex (hTR and *hTERT* mRNA), on the other hand, would be applicable to fixed tissues. However, hTR is also present at low levels in most cells lacking telomerase activity, and its level does not always correlate with telomerase activity. A better target for ISH detection would be the *hTERT* mRNA, whose levels appear to closely parallel those of telomerase activity.

For evaluating hTERT expression, several studies have reported the successful use of *hTERT* mRNA ISH (Chou et al. 2001; Kumaki et al. 2001). While it is necessary to target sequences specific to the full-length mRNA to avoid false positives resulting from splice variants of *hTERT* mRNA, the ISH detection of the *hTERT* mRNA is predicted to become a powerful tool of cancer detection. For detecting hTERT expression, immunohistochemistry (IHC) can also be used to reveal the presence of the hTERT protein in a wide variety of clinical samples, including archival paraffin-embedded specimens (Hiyama et al. 2001; Kumaki et al. 2001, 2002). In spite of its very low abundance, the hTERT protein can now be detected in paraffin-embedded samples and core biopsies with the use of polyclonal or monoclonal antibodies in conjunction with appropriate antigen retrieval (Figure 1) and/or the highly sensitive tyramide-based method of signal amplification (Frost et al. 2000). Since IHC does not require specialized equipment for detection, hTERT IHC is predicted to become a powerful new technology for cancer detection. In most cancer tissues, the hTERT protein is heterogeneously distributed and, in some cases, can display regional variability. In most cancer specimens, the signal intensity of individual hTERT-positive cells did not differ substantially between tumors with high and low telomerase activity, and the level of telomerase activity was mainly dependent on the percentage of cells displaying hTERT expression (Hiyama et al. 2001). This heterogeneity in telomerase expression appears to be an important factor dictating the overall levels of telomerase activity in tumors. With the availability of hTERT IHC, telomerase-positive cancer cells can now be detected in tissues containing a background of normal telomerase-positive cells. Likewise, Frost et al. (2000) have observed changes in the tissue distribution of the hTERT protein in cervical cancers: while the hTERT protein was limited to the lower suprabasal cells of the normal cervix, it was present at all levels of the lesional epithelium in moderate to severe dysplasia.

Telomerase in cancer prognosis and the grading of malignant tumors

In certain types of cancers, telomerase activity is upregulated during tumor progression, so that the level of telomerase activity can be used to evaluate the malignant grade of tumors and predict patient

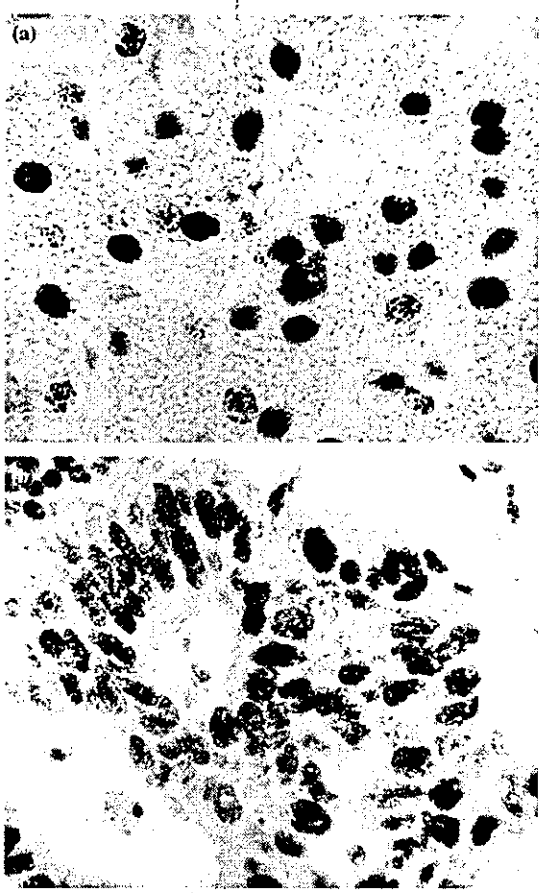

Figure 1. Immunohistochemical detection of hTERT in cancer samples. An anti-hTERT sera (EST21-A™, Alpha Diagnostic Int. Co., San Antonio, TX) was employed to reveal the presence of the hTERT protein in a FNA sample of a duct cell adenocarcinoma of the breast (a) and in a biopsy derived from an adenocarcinoma of the sigmoid colon (b). Tumor cells are revealed by the presence of brown pigments in the nucleus of hTERT-positive cells. Staining with 3,3'-diaminobenzidine (DAB) was preformed as described previously (Hiyama et al. 2001). The cells obtained from FNA sample were formalin-fixed and paraffin-embedded. For both samples, heat-based antigen retrieval was performed using a citrate buffer.

prognosis (Table 2). In certain cancers of the adults, the activation of telomerase correlates with advanced disease and poor prognosis, as in the cases of non-small cell lung cancer, gastric cancer, colorectal cancer, soft tissue tumors, and myelodysplastic disease (Chadeneau et al. 1995; Hiyama et al. 1995b; Marchetti et al. 1999; Tahara et al. 1999; Tatsumoto et al. 2000; Tomoda et al. 2002; Kido et al. 2003). In patients with colorectal cancer (Tatsumoto et al. 2000), including those undergoing curative resection of liver metastases (Smith et al. 2004), both telomerase activity and *hTERT* mRNA expression could be used as independent prognostic factors. In a retrospective study of a large number of breast cancer patients, telomerase activity correlated with a more aggressive tumor phenotype and its level was highly predictive of clinical outcomes (Clark et al. 1997). These findings suggest that telomerase activity is an useful indicator for identifying patients that would benefit from postoperative adjuvant chemotherapy.

Neuroblastomas are pediatric tumors that display a well-documented relationship between tumor biology and patient outcome. In these tumors, poor prognosis is associated with high levels of telomerase activity and full-length *hTERT* mRNA expression (Hiyama et al. 1995a; Hiyama et al. 1997a; Poremba et al. 1999b; Streutker et al. 2001; Krams et al. 2003). Interestingly, in stage 4S neuroblastoma, which represents a unique entity characterized by a high frequency of spontaneous regression, telomeres were shortened and telomerase activity was undetectable (Hiyama et al. 1995a, 1997a). Telomerase activity can also predict the outcomes of patients with gliomas, brain tumors that are consistently difficult to assign as either benign or malignant. Studies have shown that telomerase activity is present in most cases of malignant gliomas but is undetectable in grade I gliomas, making it a useful indicator of the malignant grade of gliomas (Nakatani et al. 1997; Huang et al. 1999). In tumors of the thyroid gland, telomerase activity may be useful to distinguish benign from malignant tumors and might provide a useful indicator of prognosis (Haugen et al. 1997; Saji et al. 1997). In pituitary adenoma, detection of telomerase expression may also correlate with biological aggressiveness and potential for regrowth (Yoshino et al. 2003). Telomerase activity in bone marrow has recently been reported to be a highly significant prognostic factor in pediatric patients with acute myeloid leukemia (Verstovsek et al. 2003). Collectively, these findings demonstrate that telomerase activity and hTERT expression are markers that can be used successfully to predict the outcome of cancer patients and take decisions on the appropriate treatments.

Table 2. Telomerase activity/*hTERT* mRNA as a prognostic marker.

Site	Tumor type	Correlated with prognosis	Correlated with other markers
Brain	Central nervous system malignant lymphoma; Pituitary tumor	Harada et al. (1999)[a,b] and Yoshino et al. (2003)[a]	
Head & Neck	Head & neck cancer; Oral cavity and oropharynx postchemotherapeutic tumors	Patel et al. (2002) and Ogawa et al. (1998)[a]	
Lung	Non-small cell lung cancer	Gonzalez-Quevedo et al. (2002), Hirashima et al. (2000), Marchetti et al. (1999), Taga et al. (1999)[a] and Hara et al. (2001)[b]	Dysplasia in smokers Soria et al. (2001)[b]
Breast	Invasive duct cell carcinoma		Proliferative index Carey et al. (1999)[a]; Relapse-free period Bieche et al. (2000)[a]
	Node-positive breast cancer	Clark et al. (1997)[a,c]	
Thyroid	Papillary carcinoma		Extrathyroidal extension Okayasu et al. (1997)[a]
Stomach	Adenocarcinoma	Hiyama et al. (1995b), Kakeji et al. (2001) and Usselmann et al. (2001)[a]	
Colon	Adenocarcinoma	Tatsumoto et al. (2000)[a]	Advanced stages Okayasu et al. (1998), Yoshida et al. (1999)[a], Boldrini et al. (2002), Naito et al. (2001) and Niiyama et al. (2001)[b]; Risk for metastasis Shoji et al. (2000)[a]
	Hepatic metastasis of colorectal cancer	Smith et al. (2004)	
Liver	Hepatocellular carcinoma	Kishimoto et al. (1998), Hisatomi et al. (1999) and Shimada et al. (2000)[a]	Recurrence risk (Suda et al. 1998)[a]
Pancreas	Endocrine tumors	Pearson et al. (2000)[a]	
Urogenital	Renal cell carcinoma		Tumor grade Hara et al. (2001)[a]; Advanced stage Paradis et al. (2001)[a]
	Transitional cell carcinoma	De Kok et al. (2000) and Nakanishi et al. (1999)[b]	Tumor relapse Lancelin et al. (2000)[a]
Prostate	Prostate cancer		Advanced stage Engelhardt et al. (1997)[a, b]
Uterus	Endometrial carcinoma		Recurrence risk Bonatz et al. (2001)[a]
Soft tissue	Osteosarcoma	Sangiorgi et al. (2001)[a]	Response to chemotherapy Kido et al. (2003)[a]
	Soft tissue sarcoma	Schneider-Stock et al. (2000) and Wurl et al. (2002)[a]	Recurrence and metastasis Tomoda et al. (2002)[a, b]
	Liposarcomas	Schneider-Stock et al. (2000)[b]	
Blood	Acute leukemia, B-cell Lymphocytic leukemia, Adult T-cell leukemia, Acute myelogenous leukaemia (AML)	Shay et al. (1996), Ohyashiki et al. (1997a), Uchida et al. (1999), Verstovsek et al. (2003)[a] and Xu et al. (1998)[a,b]	Recurrence in MDS Ohshima et al. (2003)[a]
	Pediatric AML	Verstovsek et al. (2003)	
	Multiple myeloma	Wu et al. (2003)	
Childhood tumor	Neuroblastoma	Hiyama et al. (1997a), Hiyama et al. (1995a), Poremba et al. (1999b)[a] and Krams et al. (2003)[b]	Cytogenetic abnormalities Brinkschmidt et al. (1998), Hiyama et al. (1997a), Hiyama et al. (1995a) and Wu et al. (2003)[a]
	Wilms tumor		Recurrent risk Dome et al. (1999)[b]
	Hepatoblastoma	Hiyama et al. (2004)[a,b]	

[a]Telomerase activity.
[b]*hTERT* mRNA.
[c]Conflicting findings have been reported.

Conclusion

In conclusion, measurement of telomerase activity and/or hTERT expression has several clinical utilities: for the early detection of cancer cells (in tumors that acquire telomerase activity at the early stages); as a prognostic indicator (in tumors that acquire telomerase activity upon progression); a marker that can distinguish malignancies from benign tumors; and a marker for detecting circulating cancer cells in the blood. *In situ* hybridization and the immunohistochemical detection of hTERT can now be used to identify telomerase-positive cancer cells in a background of non-cancerous cells. In the near future, methods for the *in situ* detection of hTERT are likely to become of common use in the clinics for both the diagnosis of cancers and the grading of malignancies.

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High-Grade Spinal Cord Tumor with Cerebellar and Retroperitoneal Extension

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A 4-month-old girl presented with a 3-week history of fecal incontinence and a 1-week history of paraparesis. She exhibited no voluntary motion of the lower extremities.

Spinal magnetic resonance imaging revealed an intradural intramedullary mass under the T9 region that exhibited a swelling cord with an area of high intensity on T1-weighted imaging (fig. 1a).

A preoperative brain CT scan 2 weeks later revealed another multiple cerebellar enhanced lesion, although the initial CT scan had not revealed any lesion (fig. 1B). The patient underwent laminotomy and posterior fossa craniectomy, and then partial excision of the spinal and cerebellar tumors. Examination of formalin-fixed, paraffin-embedded sections of the cerebellar masses showed poorly differentiated, pleomorphic cells with marked nuclear atypia and brisk mitotic activity (fig. 1C). However, the histology of the spinal tumor was not verified. Although craniospinal irradiation (50 Gy) in addition to chemotherapy was performed for 3 months, abdominal and back distension appeared. An axial body CT scan revealed an enormous retroperitoneal extension (fig. 1D). She died 2 weeks later. At autopsy, the spinal cord tumor was found to extend through the intervertebral foramen to the retro-

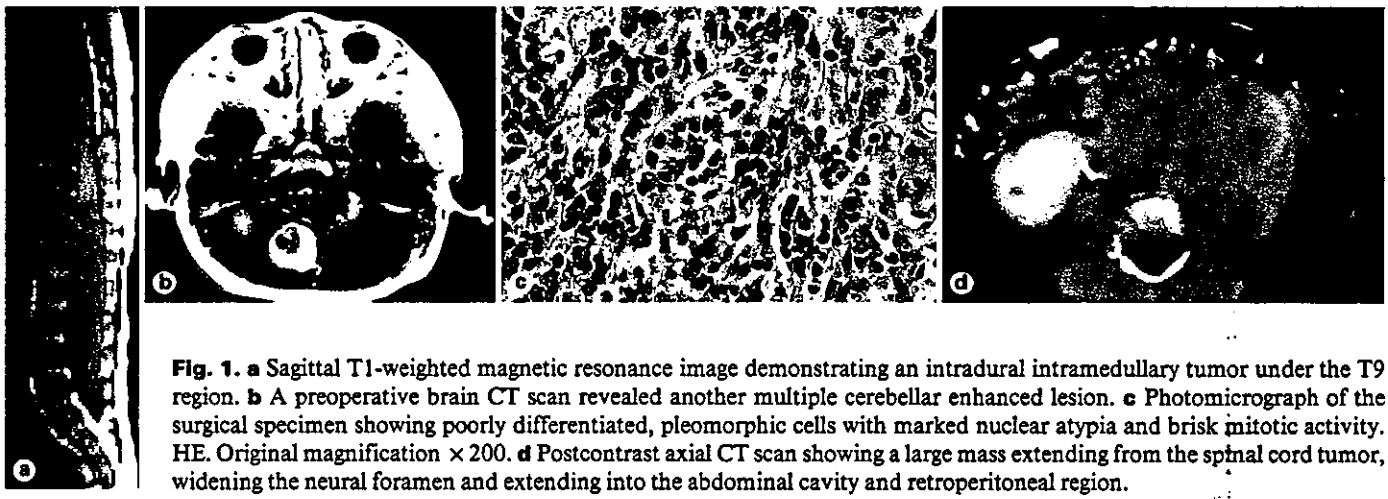

Fig. 1. **a** Sagittal T1-weighted magnetic resonance image demonstrating an intradural intramedullary tumor under the T9 region. **b** A preoperative brain CT scan revealed another multiple cerebellar enhanced lesion. **c** Photomicrograph of the surgical specimen showing poorly differentiated, pleomorphic cells with marked nuclear atypia and brisk mitotic activity. HE. Original magnification × 200. **d** Postcontrast axial CT scan showing a large mass extending from the spinal cord tumor, widening the neural foramen and extending into the abdominal cavity and retroperitoneal region.

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peritoneal region. Although the spinal cord tumor also showed the histology of a positive component with PTAH staining, the retroperitoneal tumor specimen contained rhabdoid cells which were positive on immunostaining for vimentin and epithelial membrane antigen [1].

There have been relatively few reported experiences of high-grade spinal cord tumors in the pediatric population [2, 3]. Unfortunately, these tumors invariably progress despite aggressive chemotherapy and radiotherapy. This is a rare case of a high-grade spinal cord tumor with cerebellar and retroperitoneal extension in a 4-month-old girl.

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Antitumor Activity of Gefitinib in Malignant Rhabdoid Tumor Cells *In vitro* and *In vivo*

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ABSTRACT

Purpose: **Malignant rhabdoid tumor (MRT) is a rare and highly aggressive neoplasm of young children. Current treatments have had only limited success. Epidermal growth factor receptor (EGFR) was found recently to be expressed on MRT cell lines. Gefitinib (trade name Iressa) is an oral and selective EGFR-tyrosine kinase inhibitor and has been demonstrated to be effective in inhibiting the proliferation of cancer cells *in vivo* as well as in clinical trials. This encouraged us to examine the antitumor effects of gefitinib on MRT cells *in vitro* and *in vivo*.**

Experimental Design: **The expression of EGFR in two MRT tumors and two MRT cell lines (MP-MRT-AN and KP-MRT-NS), established from these two tumor tissues, was examined by immunohistochemistry, immunofluorescence, and immunoblot. The effect of gefitinib on EGFR phosphorylation was examined by immunoblot. The effects of gefitinib on cell growth and apoptosis were examined by cell growth assay and terminal deoxynucleotidyl transferase-mediated nick end labeling assay. The *in vivo* effect of gefitinib was assessed in athymic mice that had been xenografted with MRT cells.**

Results: **The expression of EGFR was detected in both tumor tissues and cell lines. Gefitinib inhibited EGFR-phosphorylation (IC_{50} < 0.1 μmol/L) and *in vitro* cell growth (IC_{50} = approximately 10–12 μmol/L), and a high concentration of gefitinib (20 μmol/L) induced apoptosis *in vitro* (MP-MRT-AN, 42.9% and KP-MRT-NS, 47.2%). Furthermore, gefitinib at 150 mg/kg had a cytostatic effect on established MRT xenografts (MP-MRT-AN, $P = 0.039$ and 0.0014; and KP-MRT-NS, $P = 0.048$ and 0.0086).**

Conclusions: **Our results demonstrate that gefitinib has antitumor effects in MRT cells *in vitro* and *in vivo* and, thus, has promise as a novel and therapeutic strategy for MRT.**

INTRODUCTION

Malignant rhabdoid tumor (MRT) is a rare and extremely aggressive malignant tumor in childhood. It was initially described as an unfavorable histologic type of sarcomatous renal tumor, a variant of Wilms' tumor (1). Subsequently, MRT was reported to also arise from extrarenal sites, including neck, heart, chest wall, liver, pelvis, and extremities (2, 3). Despite significant advances in treatment outcome of other pediatric tumors over the past 30 years, the overall survival of renal MRT was estimated at only 26% (4). Of particular note, only 9.7% of infants that were diagnosed before the age of 12 months were alive 4 years after diagnosis (4). Therefore, new therapeutic approaches are needed.

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase and is expressed in a wide variety of epithelial malignancies including non-small-cell lung cancer, head and neck cancer, and breast cancer (5). EGFR activation promotes tumor growth by increasing cell proliferation, motility, or angiogenesis, and by blocking apoptosis (6). Gefitinib (trade name Iressa) is an oral and selective EGFR-tyrosine kinase inhibitor that blocks the signal transduction pathways implicated in proliferation of cancer cells (7). Gefitinib has been demonstrated to be effective in athymic mice studies (8–10) as well as in Phase II clinical trials for non-small-cell lung cancer (11).

About 13 MRT cell lines have been documented (12, 13). The expression of EGFR on MRT cell lines was shown by immunohistochemistry and cell growth inhibition by anti-EGFR antibody (14). However, the expression of EGFR on MRT tissues and the phosphorylation of EGFR by epidermal growth factor (EGF) on MRT cell lines have never been examined. We established two MRT cell lines from two MRT patients (13, 15). In this study, we confirm the genetic diagnoses of these cell lines to MRT by the alteration of *INI1* gene (16). Next, the expression of EGFR on two MRT tissues and two MRT cell lines established from these two tumor tissues was determined by immunohistochemistry, immunofluorescence, and Western blot. Furthermore, the antitumor effects of gefitinib on MRT cells *in vitro* and *in vivo* are demonstrated, and possible clinical applications of gefitinib for this aggressive and extremely poor prognostic MRT are discussed.

MATERIALS AND METHODS

Tumor Tissues, Cell Lines, and Cell Culture. MRT tissues were obtained from patients AN and NS in 1997 and 1992, respectively. MRT cell lines were established previously from these two patients (designated MP-MRT-AN and KP-

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MRT-NS, respectively; refs. 13, 15). MRT was confirmed in the two patients and the two cell lines by clinical, histologic, and ultrastructural examinations. Clinical data on patients AN and NS are given in Table 1. Formalin-fixed (10%) tumor tissues embedded in paraffin were used for the EGFR expressions. The A431 cell line of human epidermoid carcinoma cell line (17) was used as the positive control for the EGFR expression. Cell lines were cultured in RPMI 1640 containing penicillin, streptomycin, and 10% heat-inactivated fetal calf serum at 37°C in a 5% CO_2 incubator.

Reagents. Gefitinib was a kind gift of AstraZeneca (Macclesfield, United Kingdom). Stock solutions were prepared in DMSO and stored at −80°C. EGF was obtained from Life Technologies, Inc. (Carlsbad, CA). Stock solutions were prepared in RPMI 1640 containing 1% fetal calf serum and stored at −80°C.

Reverse Transcription-PCR Analysis. To confirm that our cell lines were MRT cell lines, we checked them for mutations in the *hSNF/INI1* (*INI1*) gene, a tumor suppressor gene for MRT (16). Mutations in this gene are specifically associated with MRT (12). In MP-MRT-AN cells, exons 1–5 of the *INI1* gene are deleted.[4] Two portions of INI1 were amplified by reverse transcription-PCR (RT-PCR), INI1CD1 (the portion corresponding to exons 1–6 of the *INI1* gene) and INI1CD2 (the portion corresponding to exons 5–9). RT-PCR was performed according to our institution's protocol (18). In brief, total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) from cultured cells and lymphocytes as positive control. Single-stranded cDNA synthesis from a 20-μg sample of template RNA with Oligo-dT (12–18) was performed using reverse transcriptase isolated from Avian Myeloblastosis Virus (20 units, Promega, Madison, WI).

RT-PCR amplification of cDNA, with corresponding 2 μg RNA, were performed in a 50-μg reaction mixture containing 10 mmol/L Tris, 50 mmol/L KCl, 200 μmol/L each of 4 deoxyribonucleotide 5′ triphosphates, 0.2 μmol/L each of primers, and 1.25 units of Taq polymerase (Takara Shuzo, Otsu, Japan). Two pairs of primers were designed to amplify the INI-1 cDNA according to the procedure of Biegel *et al.* (19): INI1CD1. forward, 5′-CTG AGC AAG ACC TTC GGG CAG-3′, and INI1CD1.reverse, 5′-GAT GGC TGG CAC AAA CGT CAG-3′; and INI1CD2.forward, 5′-AGA TCG ATG GGC AGA AGC TGC-3′, and INI1CD2.reverse, 5′-TGG AAT GTG TAC CGG GAA GGG-3′. The primer sequences of β-actin were 5′-GTG GGG CGC CCC AGG CAC CA-3′ and 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′ (13). The PCR conditions were as follows: initial denaturation at 94°C for 4 minutes, followed by 30 cycles of 94°C for 25 seconds, 66°C for 1 minute, and 72°C for 2 minutes and final extension at 72°C for 10 minutes. Analysis of PCR products was performed by 2% agarose gel electrophoresis.

Immunofluorescence. MRT cells were cultured on coverslips, fixed for 20 minutes in methanol, rehydrated in PBS, incubated with EGFR monoclonal antibody (1:80; Oncogene, Boston, MA) for 40 min, incubated with fluorescein isothiocyanate-conjugated antimouse IgG (1:80; Cappel Products, Aurora, OH) for 40 min, and observed with an Olympus BX52 immunofluorescence microscope equipped with a CCD camera (DP70, Olympus, Tokyo, Japan) as reported previously (13).

Table 1 Clinical histories of MRT patients from whom MP-MRT-AN and KP-MRT-NS cell lines were established

Characteristic	Cell line	
	MP-MRT-AN	KP-MRT-NS
Age at diagnosis	3 months	2 months
Sex	Female	Female
Site of primary tumor	Liver	Left kidney
Source of tissue	Peripheral blood	Ascetic fluid
Sampled age	10 months	9 months
Outcome	Death	Death

Histochemistry and Immunohistochemistry. Five-μm sections made from paraffin-embedded samples were stained with hematoxylin and eosin. Immunohistochemistry was performed on a section made serially to an hematoxylin and eosin-stained section. Sections were immunostained by a two-step procedure with a dextran polymer conjugate (EnVision+ system, DAKO, Glostrup, Denmark; ref. 20) following the manufacturer's instructions. Briefly, after deparaffinization and rehydration, endogenous peroxidase activity was blocked incubating the section in 0.03% hydrogen peroxide solution for 5 min. Then, all sections were incubated with Proteinase K solution (DAKO) for 5 minutes. The sections were incubate with anti-EGFR monoclonal antibody (1:200; DAKO) at room temperature for 30 min. Subsequently, slides were incubated with peroxidase-labeled polymer conjugated to goat antimouse immunoglobulin at room temperature for 30 min. The sections were incubated in 3,3′-diaminobenzidine tetrahydrochloride for 10 min to stain the antigen brown and then counterstained with hematoxylin.

Cell Growth Assay. Cells (5×10^4) were plated in normal growth medium in triplicate into 24-well cell plates. After 24 hours, cells were treated with gefitinib or DMSO for an additional 96 hours. The cells were lysed under hypotonic conditions as described previously (21), and nuclei were counted every 24 hours with a Coulter counter (22). All of the experiments were conducted on three separate occasions for each cell line.

Immunoblot Analyses. Cells were washed with Tris-buffered saline [10 mmol/L Tris-HCL (pH 7.6) and 150 mmol/L NaCl], scraped in NP40 buffer [10 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 1% NP40, 1 mmol/L sodium metavanadate, 3.3 μmol/L pepstatin, 2 μmol/L bestatin, 10 μmol/L leupeptin, 5.25 μg aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride], and incubated at 4°C for 10 minutes. Lysates were cleared by centrifugation at 4°C for 10 minutes at 20,000 × *g*. The protein concentrations of the supernatants were determined using the Lowry assay (Bio-Rad, Hercules, CA) and adjusted to equal concentrations (13).

Lysates were suspended in 2 × SDS sample buffer, boiled for 3 minutes, separated by SDS-PAGE (using equal amounts of protein in each lane), transferred to an Immobilon-P membrane, immunoblotted using anti-EGFR monoclonal antibody (1: 2500,

[4] Unpublished observations.

Transduction Laboratories), β-actin antibody (1: 2000, Sigma), anti-c-jun NH_2-terminal kinase (JNK) antibody (1:1000, Cell Signaling, Beverly, MA), anti-phosphorylated JNK antibody (1:1000, Cell Signaling), and anti- phosphorylated AKT (1:1000, Cell Signaling). Antibody binding was detected with an enhanced chemiluminescence detection system (Amersham; ref. 23).

The levels of EGFR protein were quantified with NIH Image Software 1.55 (NIH, Bethesda, MD) and were normalized against the levels of β-actin protein. Values are the mean ± SE of results from three separate experiments.

Immunoprecipitation. Lysates were prepared as described above, incubated with the appropriate amount of anti-EGFR monoclonal antibody (Transduction Laboratories, Lexington, KY) and 30 μL of protein A/G-plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, washed three times with lysis buffer, boiled in 1 × SDS sample buffer for 3 minutes, and separated by SDS-PAGE. The bands were transferred to an Immobilon-P membrane (Millipore). The membrane was blocked for 2 hours in Tris-buffered saline containing 0.5% Tween 20 with 1% bovine serum albumin, incubated with antiphosphotyrosine antibody (PY20, Transduction Laboratories) or anti-EGFR monoclonal antibody (Transduction Laboratories) for 1 hour at room temperature, washed in Tris-buffered saline containing 0.5% Tween 20, and incubated with horseradish peroxidase-conjugated antimouse IgG (1:2000, Amersham) for 1 hour at room temperature. Antibody binding was detected by enhanced chemiluminescence.

Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling Assay. MRT cells in growth medium were treated with gefitinib or DMSO. After 96 hours, adherent cells were harvested by trypsinization and pooled with floating cells. A terminal deoxynucleotidyl transferase-mediated nick end labeling assay was performed using a MEBSTAIN apoptosis kit direct (MBL, Nagoya, Japan). Fluorescein isothiocyanate-positive cells were detected with a FACSCalibur cytometer (Becton Dickinson, Mansfield, MA).

***In vivo* Antitumor Activity.** Female athymic mice (BALB/c, *nu/nu*), approximately 4–6 weeks of age, were purchased from CLEA Japan Inc. (Tokyo, Japan). This experimental procedure was approved by the Committee for Animal Research, Kyoto Prefectural University of Medicine, Graduate School of Medicine. Mice were acclimatized for 1 week before being injected with MRT cells. MRT cells, which had been resuspended in 200 μL of PBS, were subcutaneously injected into the dorsal area of athymic mice. From 1 day or 7 days after inoculation, 8 mice per group were treated for a total of 3 or 4 weeks (5 days/week) with: (1) gefitinib in 1% Tween 80, administered at 150 mg/kg/day by oral gavage, or (2) vehicles in 1% Tween 80 by oral gavage. Tumor diameters were serially measured with calipers, and tumor volumes were calculated by the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2 (9).

An *in situ* terminal deoxynucleotidyl transferase-mediated nick end labeling assay was performed as described (24). Briefly, the tumor was diced into 5 × 5-mm sections and was fixed in 4% paraformaldehyde for 48 hours, and then embedded in paraffin. The sections were deparaffinized, rehydrated, incubated in 0.03% hydrogen peroxide solution for 5 minutes to block endogenous peroxidase activity, incubated with Proteinase K solution (DAKO) for 5 minutes, incubated with reaction regent including Terminal Transferase, Biotin 16-dUTP, and terminal deoxynucleotidyl transferase-mediated nick end labeling Dilution Buffer (Roche, Indianapolis, IN) for 60 minutes at 37°C, incubated in the presence of peroxidase-conjugated streptavidin (DAKO) for 30 minutes at room temperature, incubated in 3,3′-diaminobenzidine for 10 minutes to stain the antigen brown, and then counterstained with methyl green.

Statistical Analysis. Values are expressed as the mean ± SE. The results of the apoptosis assay were compared with an ANOVA and a post-hoc Fisher's projected least significant difference test to correct for multiple comparisons. The results of the *in vivo* studies were examined using Student's *t* test. All of the *P*s represent two-sided tests of statistical significance.

RESULTS

Confirmation That Cell Lines Are MRT Cell Lines. RT-PCR failed to detect either INI1CD1 (exons 1–6 of the *INI1* gene) or INI1CD2 (exons 5–9 of the *INI1* gene) in the MP-MRT-AN cell line, and in the KP-MRT-NS cell line, it failed to detect INICD1 and detected a shortened form of INICD2 (Fig. 1). These results indicate that the *INI1* gene has a deletion and confirmed that MP-MRT-AN and KP-MRT-NS are MRT cell lines, because mutations in *INI1* gene are specifically associated with MRT (12).

Expression of EGFR in MRT Clinical Tissues and Cell Lines. We examined EGFR expression in two MRT tissues (AN and NS) from which MP-MRT-AN and KP-MRT-NS cell lines were established. EGFR immunoreactivity was diffuse in AN tissue or nodular in NS tissue, respectively (Fig. 2, *A–D*). Subsequently, the EGFR expression in two MRT cell lines was examined by immunofluorescence and Western blot. Surface EGFR expression was detected on two MRT cell lines by fluorescence microscopy (Fig. 2*E*). Over 90% of MP-MRT-AN cells and ~40% of KP-MRT-NS cells were strongly stained.

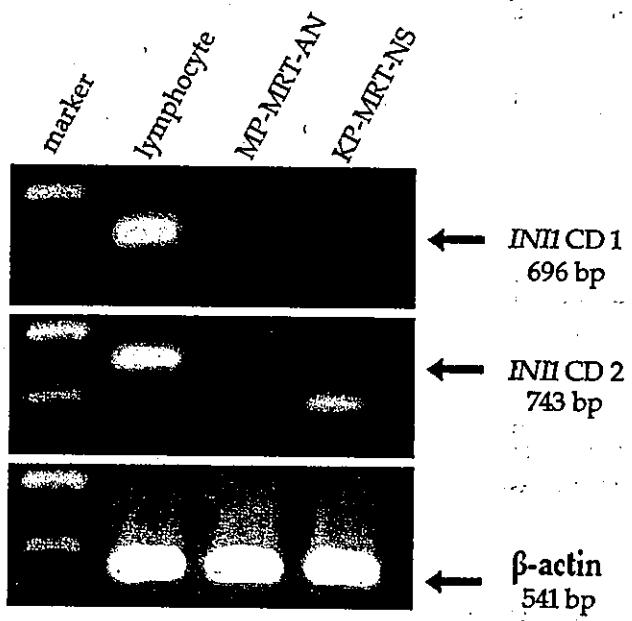

Fig. 1 RT-PCR analysis of *INI1* gene expression in MRT cell lines.