Thymidine kinase 1 as a tumor biomarker: technical advances offer new potential to an old biomarker

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Thymidine kinase 1 (TK1) is a key enzyme in DNA precursor synthesis. It is upregulated during the S phase of the cell cycle and its presence in cells is an indicator of active cell proliferation. In studies since the 1980s, TK1 has been shown as a clinically valuable biomarker for the management of hematological malignancies. However, TK1 activity assays may underestimate serum TK1 in subjects with solid tumors limiting its sensitivity. The development of TK1 immunoassays has made the assay of TK1 more widely available and increased its applicability to solid tumor diseases. This paper will review TK1 as a tumor biomarker with emphasis on recent studies and technologies plus highlight its potential in drug discovery and as a therapeutic target.

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Cell proliferation: what really matters

Although there are many biomarkers that are related to cell mass and which can be used as an aid to diagnosis, for example PSA in prostate cancer [1] and that can aid in diagnosis and therapy selection, for example BRCA [2], there are few serological biomarkers that indicate the rate of cellular proliferation. Proliferation biomarkers are forward-looking biomarkers and as such could provide especially valuable information for making prognosis and therapy choices. They complement biomarkers of tumor mass as will be described below.

Proliferation biomarkers are proteins that are expressed in proliferating cells and are routinely detected using immunohistological techniques. Such biomarkers include, PCNA, Ki-67 and TK1 [3,4]. These are expressed during the cell cycle in proliferating cells. Ki-67 is the most widely used biomarker of this type and it is expressed during the G1, S, G2 and M phases of cell cycle.

Immunohistological techniques have the drawbacks of being labor intensive, requiring biopsy material and being somewhat subjective. Furthermore, only a small part of the total tumor mass is studied. A serological test that could provide an indication of the total rate of proliferation and disruption would be more convenient and provide unique and valuable information.

TK1 is unique as a proliferation biomarker as it can be determined both immunohistologically in cells and in body fluids using enzyme activity assays and immunoassay.

This article will review recent advances in the application of TK1 as a biomarker and speculate as to how it may be applied in the future development of cancer monitoring and therapies. Emphasis will be laid on the use of immunoassays. Excellent reviews of the earlier literature are available [3,4].

Biochemistry & physical properties of TK1

DNA precursor synthesis

Thymidine kinases are essential for coupling a phosphate group from ATP to thymidine as a part of the DNA synthesis and repair machinery in all living cells.

There are two major pathways involved in DNA precursor synthesis; the *de novo* pathway and the salvage pathway. In the *de novo* pathway, purine and pyrimidine nucleotides are synthesized from low molecular weight precursors, whereas the salvage pathway recycles deoxyribonucleosides (dNs) from degraded DNA. TK1 is a dNs kinase involved in the utilization of thymidine as a part of the salvage pathway [5].

Thymidine kinase activity is present in cells as two isoenzymes: TK1 and TK2.

TK1 converts deoxythymidine (dT) to deoxythymidine monophosphate (dTMP) by transferring a phosphate from ATP. The monophosphate undergoes further phosphorylation to deoxy thymidine triphosphate (dTTP) that is incorporated into DNA. dTTP self-regulates DNA precursor synthesis by negative feedback of TK1 [6].

TK1 activity is cell-cycle regulated and shows a different pattern of activity in normal proliferating cells compared with tumor cells. In normal proliferating cells, TK1 activity reaches peak at late G1 phase/early S phase and is dramatically reduced to undetectable levels by the end of M phase. TK1 activity may be 15-fold higher in S/G2 compared with G1 [7]. However, TK1 activity may remain elevated in G2 and M phases of cell cycle in malignant cells [8], most likely due to disordered regulation of transcription and degradation. TK2 activity is quite stable without any fluctuations during the cell cycle since it is located in and needed for DNA synthesis in mitochondria [9]. The presence of TK1 in cells is associated with DNA synthesis and cell proliferation, but it is not specific for malignant cells [3,4].

Anticancer therapy, for example, 5-fluorouracil, may suppress the *de novo* pathway by inhibiting thymidylate synthase providing an advantage for cells more able to use the salvage pathway [10] which may be an explanation for the link between high expression of TK1 and more aggressive malignant disease.

TK1 in DNA repair

When DNA is damaged by radiation or chemotherapeutic agents, significant increases in TK1 levels are found [5,11]. This may increase the efficiency of the recycling of thymidine released during DNA breakdown. TK1 is involved in DNA repair and depletion of TK1 during DNA recovery results in cell death [5].

Molecular forms of TK1

TK1 is found in a range of forms depending on the source and purity. The basic intracellular form of TK1 is a homo dimer with a subunit size of 25 kDa. The monomer lacks enzyme activity, but in the presence of ATP and/or at high concentrations it polymerizes to dimers and the most active tetramers with a molecular weight of approximately 100 kDa [6]. The intracellular TK1 dimers can be rapidly converted to the active tetramer in the event of DNA damage, in advance of new synthesis [12].

TK1 is released from cells that die during proliferation; the concentration of TK1 in extracellular fluid is a measure of disruption of dividing cells. Normal cells seldom disintegrate during proliferation and it usually only occurs during accelerated or unregulated proliferation, as in malignancies. Following its release, TK1 forms complexes of differing molecular weights and enzyme activities, a factor which is of importance regarding the development of assays.

Cellular TK1 from human leukemia cells eluted mainly as tetramers (100 kDa) with a minor peak around 700 kDa [13,14]. However, serum TK1 from a leukemia patient eluted as a high MW complex around 700 kDa plus a minor peak of low molecular weight [14,15]. Analysis of molecular forms of serum TK1 from breast cancer and prostate cancer patients has demonstrated the existence of active TK1 as complexes ranging in size from 720 to 200 kDa. Furthermore, immunoblot analysis of serum TK1 showed that it also included low molecular weight complexes that lacked enzyme activity [15].

TK1 assays

Because of its close association with DNA synthesis and cell proliferation, TK1 is a valuable biomarker for studying malignancies. TK1 has been quantified by two different technologies: by measuring its enzyme activity or its mass, via immunoassay. A brief description of the technologies is given below and summarized in Table 1.

Intracellular active TK1 is a tetramer but upon release it polymerizes producing high molecular weight complexes with a range of molecular weights and enzyme activities (see Figures 1 & 2) [15]. Furthermore, serum may contain

enzyme inhibitors [16]. These factors provide challenges to the assay of TK1, particularly for the forms associated with solid tumors [15].

Enzyme activity-based assays

TK1 activity assays are based on the transfer of phosphate groups from ATP to labeled TK1 substrates resulting in labeled monophosphate derivatives (dTMP) which are detected by a variety of methods.

TK-radio enzymatic assay (TK-REA)

TK-REA uses ^{125}I -labeled deoxyuridine as a substrate. In brief, radioactive substrate is added to the sample and incubated at 37°C . TK1 in the sample converts the labeled substrate to ^{125}I -deoxyuridine monophosphate (dUMP). Then the reaction product (dUMP), but not the unconverted substrate, is absorbed by a TK-REA separator tablet. After washing, the radioactivity in the sedimented particles is measured and TK1 levels expressed as U/l [17]. The higher the radioactivity the higher the TK1 activity.

TK-Liaison assay

This assay utilizes a two-step procedure where in the first step, TK1 in the sample phosphorylates 3-azido-3-deoxythymidine (AZT) to the corresponding 5-monophosphate (AZTMP).

In the second step, the generated AZTMP is detected utilizing a competitive ELISA technique. In brief, the sample is incubated with goat anti-AZTMP antibodies and HRP-labeled AZTMP in a microtiter plate coated with an excess of rabbit anti-goat antibodies. Competition between the generated AZTMP and the HRP-labeled AZTMP occurs for the primary goat antibody. The goat antibodies, with bound AZTMP or HRP-labeled AZTMP, are captured by the anti-goat antibodies on the microtiter plate. Unbound HRP-labeled AZTMP is washed away, and the bound HRP-labeled AZTMP is quantified using TMB as a substrate. The color developed is inversely proportional to the initial TK1 activity and is expressed in U/l [18].

[^3H]-deoxythymidine phosphorylation assay

The assay is based on the use of tritiated deoxy-thymidine, a natural substrate for TK1 instead of substrate analogs [19]. It may be considered the 'gold standard' for determining TK1 activity. In brief, samples are incubated with a reaction mixture containing ATP, DTT, MgCl_2 and ^3H -labeled deoxy-thymidine. TK1 in the sample converts the labeled substrate into tritiated deoxy thymidine monophosphate (^3H -dTMP). The product of the reaction is spotted onto DE-81 filter papers and the filter papers allowed to dry. The filter paper is then washed with ammonium formate to remove the unconverted substrate. The bound ^3H -dTMP is eluted from the filter papers using HCl and KCl buffer and its radioactivity measured using β -scintillation counter. The activity is expressed as pmol/min/ml. The higher the radioactivity, the higher the TK1 activity in the sample.

Divitum assay

The Divitum TK1 enzyme activity assay utilizes bromodeoxyuridine (BrdU) as a substrate. In this assay, TK1 in the sample phosphorylates BrdU to bromodeoxyuridine monophosphate (BrdUMP). This BrdUMP undergoes further phosphorylation by added yeast enzymes and the formed bromodeoxyuridine triphosphate (BrdUTP) is incorporated, via a DNA polymerase, into DNA strands that are bound to a microtiter plate. The BrdUTP bound to the plate is then quantified using a bromodeoxyuridine antibody conjugated with alkaline phosphatase. The color developed is proportional to the amount of bromodeoxyuridine monophosphate formed by TK1 and is, therefore, proportional to the initial TK1 activity. TK1 activity is expressed in Divitum units/l (Du/l) [20]. The Divitum assay is optimized for measuring TK1 activity in high molecular weight complexes and may be less sensitive than the Liaison method for measuring low MW TK1 complexes [21], those that also predominate in serum from subjects with solid tumors.

Immunoassays

The development of antibodies against different exposed regions of TK1 demonstrated the potential for the development of highly effective TK1 immunoassays. Moreover, immunoassays widen the clinical applications of TK1 for diagnosis and treatment monitoring of different solid tumors by enabling determinations of TK1 forms that lack enzyme activity.

TK1 immunoassays

Several attempts have been made to develop TK1 ELISAs suitable for determining TK1 concentrations in serum samples [22], but none of these are widely available.

One reason is the need for antibodies of appropriate specificities, in particular the development of antibodies against the exposed '210' epitope covering the C-terminal amino acid sequence 194-225 [23]. Two assays have been developed based on antibodies against this epitope; a direct dot-blot assay with chemiluminescence end point [24] and a microtiter sandwich ELISA [25].

Dot-blot chemiluminescence assay

This dot-blot assay is a nitrocellulose membrane based assay with a chemiluminescent substrate utilising a primary chicken IgY antibody directed against a C-terminal TK1 epitope and a secondary labeled anti-IgY antibody. In brief, the sample is spotted onto a nitrocellulose membrane where the proteins in the sample bind. After blocking, the membrane is incubated with a primary anti-TK1 antibody which binds to the TK1 on the membrane. After washing, a biotinylated second antibody directed against IgY antibodies is added followed by streptavidin labeled HRP and a chemiluminescent substrate. The light intensity of the dots is measured by a CIS-II imaging system. The greater the emission of light, the higher the TK1 concentration in the sample. TK1 levels are expressed as pM [24].

TK 210 ELISA

A problem in the development of TK1 assays has been that TK1 circulates in serum in complexes of differing molecular weights and enzyme activities. Furthermore, the molecular weight distributions of these complexes varies between different types of malignancies [15].

Figure 1. TK1 complexes, immunoreactivity and enzyme activities in serum from a subject with myelodysplasia.

The AroCell TK 210 ELISA system has reduced this problem by pre-incubating the sample with a buffer containing agents that breakup the high molecular weight complexes. Following this pretreatment of the sample, the TK1 is assayed using a standard microtiter ELISA technique. In brief, the treated samples are added to a microtiter plate coated with anti TK 210 monoclonal antibodies. After incubating and washing, a second anti-TK 210 antibody labeled with biotin is added. After further washing, the color is developed with streptavidin labeled horse radish peroxidase with TMP as substrate. The stronger the color generated, the higher the TK1 concentration in the sample. The values are expressed as $\mu\text{g/l}$ [25].

Figure 2. TK1 complexes, immunoreactivity and enzyme activities in serum from a subject with prostate cancer.

Table 1. The table below is intended as a broad overview without recommending any particular assay method.		
Assay variable	TK1/TK 210 immunoassays	TK1 enzyme activity assays
Applicability	+ Greater sensitivity in subjects with solid tumors [16].	– Not all TK1 is enzymatically active in sera from subjects with solid tumors [15].
	+ More consistent TK1 values in different disease types.	– Specific activity of serum TK1 may differ between disease types [15].
	– Potentially, a different antibody for each species.	+ Potentially applicable to many animal species and cell lines.
	+ Species specific differences in TK 210 antigen may make it possible to distinguish TK1 from host and implant in xenograft studies.	– Source of TK1 activity may be unclear in xenograft studies.
	+ Probable good correlation with activity assays in sera from hematological malignancies.	+ Probable good correlation with immunoassays in sera from hematological malignancies.
	+ Potential to use the same antibody for immunohistology.	–
	+ Probable good correlation with activity assays in cell extracts.	+ Probable good correlation with immunoassays in cell extracts.
Confounding factors	+ Defined reaction; less assay interference.	– Serum may contain enzyme inhibitors [16].
	– Antibody dependent.	+ Not dependant on a specific antibody.
	– Differences in unitage between antisera.	– Differences in unitage between substrates.

Figures 1 & 2 show examples of the relationship between the presence of the TK 210 epitope and enzyme activity in different molecular weight fractions in serum from subjects with different malignancies. Note that in a sample from a subject with a hematological malignancy there is a good agreement between the presence of the TK 210 epitope, as shown by Western blotting, and enzyme activity whereas much of the TK 210 in a subject with a solid tumor was associated with low molecular weight fractions with low or no activity [15]. This finding helps to explain the predominance of studies on the application of TK1 activity assays being in the field of hematological malignancies. The pretreatment of the samples may make comparisons between subjects and types of malignancies more reproducible. The development of immunoassays based on the TK 210 antigen opens new possibilities for the study of TK1 in solid tumors and, most likely, also in different forms of hematologic malignancies.

TK1 in biomarker studies

The rate of cellular proliferation and disruption is an important parameter usually monitored by immunohistological studies on biopsies. The ‘gold standard’ is the proliferation index which is the % of cells staining for Ki-67. Tissues that have a high Ki-67 proliferation index tend to also have a high proliferation index based on TK1 staining, although the same cells do not necessarily stain for both biomarkers [26,27]. TK1 staining may be better than Ki-67 at discriminating between breast cancer stages [28].

However, the main advantage of TK1 as a proliferation biomarker is being accessible in serum and, therefore, providing an indication of the total disease activity in the subject. In this, it may be unique as a serological biomarker for cell proliferation and disruption.

TK1 in population screening

Serum TK1 may be elevated in many situations where increased cell proliferation may be occurring. This includes a range of conditions including infections, inflammatory conditions and (pre) malignancies [3,4,17]. A large-scale population screen utilizing a dot-blot chemiluminescent assay found that 83% of subjects with an elevated serum TK1 (>2 pM, highest 1.1 percentile) had an increased risk of having a pathological condition, 22% with viral infections or inflammation and 61% malignancies [29]. An elevated serum TK1 may be a signal for further clinical investigation, without indicating the cause.

TK1 & hematological malignancies

Probably because most of the serum TK1 in hematological malignancies is enzymatically active (see Figure 1), serum TK1 has been found to be a valuable biomarker in the management of hematological malignancies. Furthermore, lymphocytes from subjects with lymphomas probably express a higher level of TK1 than those from normal subjects [30].

Studies have shown that in hematological malignancies, serum TK1 activity levels:

- are associated with clinical stage;
Subjects with apparently early clinical stage disease but with a relatively elevated serum TK1 activity had a tendency for disease progression [31];
- are associated with tumor burden [30];
- correlate with expression of the proliferation biomarker Ki-67 [31,32];
- may correlate with the total mass of proliferating cells (Ki-67 index $\times$ tumor mass) [30];
- may add value to other tumor-related biomarkers [33];
- can aid in making prognoses [33–35];
- may be a biomarker of disease progression [36].

There have been, as of yet, fewer studies on hematological malignancies where serum TK1 has been determined using immunoassays. However, TK1 measurements using a dot-blot chemiluminescence assay prior to and after therapy were shown to correlate with clinical response and therapy in non-Hodgkin's lymphoma [37] and aid in making prognoses in subjects with chronic lymphocytic leukemia [38].

Furthermore, serum TK1 levels measured using a TK 210 immunoassay correlated closely with TK1 activity levels measured using the tritiated thymidine assay and they gave a similar receiver operating characteristic (ROC) curve (0.84 for TK 210 ELISA, 0.79 for ^3H -deoxythymidine phosphorylation assay) for distinguishing between subjects with and without hematological malignancies [39]. Preliminary studies indicate that immunoassay of TK1 using the TK 210 antibody can distinguish between healthy subjects and those with hematological malignancies and possibly aid in disease monitoring [40,41].

The close agreement between results with activity and immunological assays is valuable in that it enables results with more modern assays to be compared with the historical data. For further references on this topic, the reader is referred to references [3,4].

TK1 in the study of solid tumors

There have been fewer studies on the use of serum TK1 activity to study solid tumors although there are many studies showing increased expression of TK1 in solid malignancies [3,4] and even in pre-malignant conditions [42]. This may be due to the greater difficulty of measuring TK1 activity in serum from subjects with solid tumors as mentioned previously. This review will mainly concentrate on results obtained using immunoassay with reference, where appropriate, to studies performed with other methods.

Elevated serum TK1 activities or immunological concentrations have been found in many solid tumors including gastric [43], ovary [44], cervical [45], esophageal [46], lung [22,47], prostate [15,48] and breast [15,42]. Elevated serum TK1 levels post surgery have been associated with poorer outcome [49].

Figure 3. Receiver operating characteristic analyses of serum TK 210 ELISA and TK1 activity assays for distinguishing breast cancer patients from healthy subjects. AUC: Area under the curve.

Figure 4. Receiver operating characteristic analyses of serum TK 210 ELISA and CA15-3 for distinguishing breast cancer (T2) patients from healthy subjects.

Studies have shown that in solid malignancies, serum TK1 immunoassay levels:

- are associated with clinical stage [25,49];
Subjects with apparently early clinical stage disease but with a relatively elevated serum TK1 activity had a tendency for disease progression [49];
- may add value to other tumor-related biomarkers [25,48,50];
- can aid in making prognoses;
Elevated serum TK1 levels post surgery and chemotherapy have been associated with poorer outcome [49];
- may be a biomarker of disease progression [49].

The greater sensitivity for immunoassay in studying solid tumors may be explained by the occurrence of enzymatically inactive TK1 forms in the serum of subjects with solid tumors [15]. Figure 3 compares the diagnostic accuracy (ROC analyses) of TK1 an enzyme activity assay and TK 210 ELISA for the diagnosis of stage II breast

cancer. The TK 210 ELISA was nearly twice as sensitive (0.50 vs 0.26) with a similar specificity and a higher AUC (0.90 vs 0.79) [25]. Other studies have shown TK1 immunoassay to be more sensitive than enzyme activity assays in studying solid tumors [16,47].

TK1 immunoassay can be valuably combined with other biomarkers. Jiang *et al.* found that adding TK1 immunoassay to a panel of biomarkers increased the diagnostic accuracy for lung cancer [50]. Similarly, the addition of TK 210 ELISA to CA15-3 increased the ROC value for diagnosing breast cancer (Figure 4) [25] and its addition to pro-PSA and the prostate health index increased the ROC value for the diagnosis of prostate cancer [48].

TK1 as a therapeutic target

TK1 is not essential for normal cell growth but it is important in the repair of DNA damage such as may be caused by chemotherapy [6]. Furthermore, upregulation of TK1 can reduce the efficacy of chemotherapy, for example by 5-fluoro-2'-deoxyuridine (5FdUrd) [10].

Anticancer drugs (e.g., FdUrd) may inhibit the *de novo* pyridine pathway which leads to toxicity for normal tissue while, possibly, selecting for malignant tissues with a higher TK1 expression.

Drugs that require TK1 for activation have, therefore, the potential to be less toxic to normal cells while targeting malignant tissues with higher TK1 concentrations.

This has been shown using a radioiodine labeled thymidine derivative, [$^{123/125}$ I]-5-iodo-4' thio-2'-deoxyuridine, which is incorporated into DNA resulting in cell death. To increase the incorporation of this radioactive thymidine derivative into DNA, *de novo* synthesis of thymidine was blocked via the application of the thymidine synthetase inhibitor FdUrd [51].

Boron (^{10}B) has the property of emitting very short-range radiation when bombarded with neutrons killing the cells in which it is found but sparing surrounding tissue. Several boron labeled thymidine derivatives have been developed and shown to be valuable in the radio-therapy of tumors, especially gliomas. The higher TK1 content of proliferating tumors may make them particularly sensitive to ^{10}B assisted radiotherapy. For example, in a study comparing TK1 knock-out and wild-type tumors implanted into rats, the wild-type (TK1+) tumors took up about 2.5-times more boron than the TK1- knockout tumors. Animals bearing TK1+ tumors had an average 15-fold reduction in mean tumor volume on day 30 after implantation compared with TK1- tumors [52].

TK1 has been reported to be expressed on the surface of Burkitt's lymphoma and acute lymphoblastic leukemia cells, but not on healthy cells, making it a potential target for immunotherapy [53].

Assays determining TK1 concentrations in tumor tissues and, maybe, in serum could help to select those subjects most likely to benefit from these novel types of therapies.

TK1 in positron imaging

Positron [^{18}F] labeled thymidine derivatives (e.g., 3'-deoxy-3' [^{18}F]-fluorothymidine [[^{18}F]FLT]) offer a means of imaging tumor mass and tumor proliferation. These compounds are phosphorylated by TK1 and are then trapped in the cells [54]. That malignant cells tend to have higher concentrations of TK1 makes these derivatives particularly attractive.

In general, the uptake of [^{18}F] thymidine derivatives correlate better with the proliferation index in cell lines with greater dependence on the salvage pathway [55].

Therapy with agents that suppress the *de novo* pathway (e.g., hydroxyurea) or cause DNA damage may cause upregulation of TK1 which leads to an initial increase in [^{18}F]FLT uptake (flare) [12,56] followed by a decline as cell death occurs. Uptake may also differ according to the dependence of the tumor on the *de novo* or salvage thymidine pathways [57]. Thus, positron imaging with [^{18}F] thymidine derivatives could potentially provide information as to the mechanism and time course of therapeutic effects, but it could also mean that the optimal time to image a tumor may vary according to the mechanism and pharmacokinetics of the agent [56]. [^{18}F] thymidine derivatives uptake can be used to study cell proliferation in both *in vitro* culture [12,58] and xenografts [59] making it a translatable biomarker.

Thymidine kinase in drug discovery and development

In vitro studies

A crucial stage in drug discovery is *in vitro* validation of potential new compounds.

TK1, being involved in DNA synthesis and repair [5], provides an *in vitro* means of measuring the effects of drugs that target these pathways, TK1 immunohistology could aid in the selection of cell lines with different expression of TK1 which may respond differently to anti-metabolites [60].

TK1 immunoassay in cell culture can be used to follow dose–effect curves of drug–cell interactions. For example, in a leukemic cell line treated with doxorubicin, low doses of doxorubicin induced intracellular TK1 concentrations but at higher drug levels it decreased and, simultaneously, TK1 appeared in the culture medium as cell death occurred [61]. These patterns match what is known about doxorubicin, in that it damages DNA and that TK1 is involved in DNA repair. Furthermore, this pattern is similar to that seen also in positron imaging where an increased uptake of positron labeled [^{18}F] thymidine analogs occurs in the early stages of therapy (flare) then declining as treatment progresses [57]. This may be particularly observed with inhibitors of the *de novo* pathway [12].

Since TK1 is involved in DNA repair and resistance to chemotherapy, increases in TK1 may not be seen if compounds which suppress cell proliferation by other means – for example, anti-hormonal therapy or CDK4/6 inhibition, are used. This has been shown for the CDK 4/6 inhibitor Palbociclib [62] and anti-estrogen therapy [63] using the Divitum enzyme activity assay.

Very few cells are needed for a study and together with the ability to automate ELISA procedures, new compounds can be rapidly and economically tested. Furthermore, TK1 is an early indicator of cellular proliferation and may be upregulated earlier than Ki-67 and before BdrU incorporation into DNA occurs [64].

Xenograft studies

Potential therapeutic compounds are often studied using tumor xenografts in immune-deficient mice. TK1 activity assays will measure TK1 derived from both the human graft and the host. However, the human TK 210 antigen differs from that in mice, potentially, enabling better discrimination of the effects of the test substance on the tumor graft than using TK1 activity assays.

Clinical studies

In human clinical trials, serum and tissue TK1 could provide a means to stratify patients/subjects according to the clinical stage of their disease and the rate of cell proliferation and disintegration [25,46]. This could enable clearer discrimination between study groups and also clearer definition of the treatment effects.

Elevated tissue TK1 values are associated with a poorer therapeutic prognosis [65] and may provide a guide to therapeutic sensitivities, for example, subjects with a high tumor TK1 responded less well to tamoxifen therapy [65].

During therapy, serum TK1 levels may fluctuate, due to destruction of tumor cells but also, possibly, due to the proliferation and destruction of normal cells as a part of an inflammatory response or general cytotoxic effect [66]. This is supported by findings of elevated serum TK1 levels following surgical removal of tumors [67]. In general, therefore, a therapeutic response is associated with an increase in serum TK1 concentration during and immediately after therapy. However, approximately 3–5 weeks after completed successful therapy TK1 concentrations should be reduced to near normal concentrations [37,68]. A much faster apparent decline may be seen if TK1 enzyme activity is measured [69]. This decline could possibly be due to the presence of TK1 inhibitors in serum that inhibit its activity but leave it open for immunoassay [16].

If serum TK1 protein is high and/or increasing it is often associated with relapse or therapeutic escape [36].

Compounds that are not directly cytotoxic but reduce cell proliferation may reduce TK1 levels. Check point inhibitors that prevent cells entering the S phase of cell proliferation may inhibit TK1 production, therefore, a positive response could be linked with a fall in TK1. This was shown for the CDK4/6 inhibitor Palbociclib using the Divitum TK1 activity assay [62]. Similarly, successful anti-hormonal therapy of breast cancer may be associated with a fall in serum TK1 activity, with decreased TK1 activity levels after a month being associated with better outcome [63].

Translational biomarker

A critical parameter in studying the effects of anticancer therapy is the proliferation rate of the malignancy. Assays of tissue and serum TK1 can be used to study the effects of compounds in cell-culture, potentially in xenografts and in human clinical trials making it a translational biomarker.

Future perspective

The development of better immunoassay techniques for TK1 based on the 210 epitope opens new possibilities for the application of this biomarker.

The development of anticancer therapies and tumor biomarkers are being rapidly advanced due to greater understanding of the genetic and biological factors that stimulate and inhibit tumor growth. The rapid progress in cancer immunotherapy is offering new hope and possibilities in treatment of previously incurable malignancies, but the very high cost of novel therapies, often in the range of hundreds of thousands of dollars for a single patient, plus the risk of side effects, are necessitating improved patient selection. Thus, biomarkers will have an increasingly important role in future personalized medicine.

However, there is a problem in that there is a lack of serological biomarkers that can provide forward-looking information regarding the effects of therapy (prognostic biomarkers). For example, the American Society of Oncologists wrote that *"No biomarkers for monitoring advanced breast cancer are available"* [70] and in prostate cancer, the European Association of Urology have stated *"PSA is a less reliable biomarker in monitoring advanced metastatic disease"* [71].

TK1 has great potential here since it is a biomarker for the turnover of proliferating cells and may aid selection of the patients with aggressive disease and most in need of therapy. Thus, a TK1 protein assay can be said to 'measure what really matters'. It could save considerable resources and avoid unnecessary side-effects in those less likely to benefit from a particular therapy.

Since most malignant cells tend to express TK1 at higher and unregulated concentrations than normal cells, TK1 has the potential to monitor therapies across a wide range of cancer types and therapies.

TK 210 immunoassays increase the range of malignancies that can be studied with this biomarker, plus immunoassays are more compatible with standard laboratory assay platforms. This simplifies the combining of TK1 assays with other biomarkers leading to the development of new and better algorithms for patient diagnosis and monitoring.

The relative overexpression of TK1 in proliferating cells makes TK1 an interesting target for therapeutic intervention and imaging probes. The role of TK1 in DNA damage repair suggests that its assay could add particular value to the study of anti-metabolic agents and indicates its potential as a complementary diagnostic.

Cell proliferation and disruption are key parameters in oncology, TK1 immunoassays offer new, improved means of monitoring these essential processes, in model systems pharmaceutical research and clinical practice.

Executive summary

TK1 is a well-validated biomarker for studying malignancies. However, it is not widely applied due to complicated and closed assay systems and lack of studies utilizing recent analytical methods. However:

- the immunoassay of TK1 increases its value as a biomarker.
- the immunoassay of TK1 makes it a more sensitive and novel biomarker for studying solid malignancies.
- TK1 levels in malignancies are associated with clinical stage offering potential for patient and subject stratification.
- elevated and/or increasing serum TK1 levels after treatment indicate therapy escape.
- TK1 may be a particularly valuable biomarker for monitoring therapy with cytotoxic agents.
- TK1 can be used as a translational biomarker in drug discovery.
- changes in cell culture seem to be similar to those seen *in vivo* and may offer a new biomarker for studying anti-proliferative agents.
- TK1 has great potential as a complementary biomarker.
- changes with disease status and response are associated with changes in TK1 levels.
- TK1 may have potential as a target for novel therapeutics and imaging agents.
- there is need for better biomarkers to follow therapy response.
- TK1 levels are associated with changes in the disruption of proliferating cells.
- TK1 may be profitably combined with other biomarkers to improve diagnostic accuracy.

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