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Thymidine Kinase 1: Diagnostic and Prognostic Significance in Malignancy

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Doctor of Philosophy

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ABSTRACT
Thymidine Kinase 1: Diagnostic and Prognostic Significance in Malignancy

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Thymidine kinase 1 (TK1) is a cancer biomarker which has diagnostic and prognostic potential in a variety of malignancies. TK1 is significantly elevated in the serum and tumor tissue of most malignancies. This increase in TK1 can be detected in the very early stages of malignancy, including in pre-malignant disease with an increased risk for progression. Several studies have demonstrated that elevated TK1 is found in serum months before any clinical symptoms of malignancy. It has also been demonstrated that TK1 is elevated months before clinical recurrence of malignancy.

This work first sought to demonstrate the early nature of TK1 expression in breast tumor tissue and pre-malignant tissue. We found that TK1 is elevated in breast hyperplasia tissue and breast carcinoma tissue. In this study we also identified some cases of 'normal' tumor margins (considered normal by current pathological standards) which also had elevated TK1 expression. Conversely, true normal breast tissue from noncancerous individuals had no reported elevation in TK1 expression. This study illustrated that TK1 is elevated in pre-malignant breast hyperplasia tissue, as well as some 'normal' tumor margins. TK1 expression was significantly elevated in lung, prostate, colon, esophagus, stomach, liver, and kidney tissues. This work further investigated TK1 expression in a variety of malignant tissue including the two leading causes of cancer mortality in men: lung and prostate cancer. In our study, TK1 was significantly elevated in lung and prostate cancer but not significantly elevated in prostate hyperplasia tissue. TK1 expression also increased with increasing grade in prostate carcinoma tissue. Overall, this work demonstrated that TK1 is a good universal marker of malignancy and is elevated in early cancer development.

Despite the potential for TK1 as both a screening and monitoring treatment tool, there have been significant challenges associated with developing a clinically relevant method of TK1 detection. This work proposes one clinically relevant method of detection, namely a TK1 ELISA. Using preoperable lung cancer patients and normal controls, we developed a sensitive and specific ELISA which shows highly statistically significant differences in serum TK1 levels between stage 1 and stage 2 lung cancer compared with normal controls. In fact, this TK1 ELISA is more sensitive and accurate than the traditional TK radioassay, which was unable to detect differences in TK1 between early stage lung cancer and normal patients. Although elevated TK1 is not lung cancer specific, we reported significantly elevated TK1 levels in lung cancer sputum. Screening of sputum and serum for TK1 may be one method for the early detection of lung cancer.

Overall, we report TK1 has promising diagnostic potential in a variety of malignancies. We also propose one sensitive and specific method to detect TK1 levels which may easily be adapted to meet current clinical applications. We hope this work will help propel TK1 forward into clinical view in the coming years.

Keywords: Thymidine Kinase 1, lung cancer, proliferation marker, ELISA

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Introduction: The Clinical Significance and Biology of Thymidine Kinase 1

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Abstract: Cancer is a complex disease in which there is still a demand for clinically relevant biomarkers. Thymidine Kinase 1 (TK1) is one cancer biomarker which has value both in early detection and monitoring treatment responsiveness. TK1 is primarily responsible for maintaining thymidine nucleotide pools by converting dT to dTMP. In malignancy, TK1 plays a crucial role in DNA damage helping cancer cells to repair themselves following chemotherapeutic treatment, a potential mechanism of resistance. The structure of TK1 is essential to its function and regulation in malignancy. A thorough understanding of these aspects helps to explain the challenges associated with TK1's detection and its transition into the clinic. In malignancy, TK1 is elevated in both the serum and tumor tissue. This is true for a wide variety of malignancies including both haematological and solid tumors. Typically its elevation is correlated to stage and grade of a tumor. High levels of TK1 are indicative of a worse prognosis and greater risk of recurrence. In fact, numerous studies have revealed that serum-TK1 levels are elevated months before a patient exhibits clinical relapse. In comparison studies with other proliferation markers and other serum biomarkers, TK1 appears to be more accurate and sensitive. Additionally, TK1 has therapeutic potential and has begun to be used as an imaging tool for positron emission tomography (PET). As TK1 is propelled into clinical view, it becomes more essential to understand the basic biology and clinical advances associated with this promising biomarker. This review seeks to highlight these important advances to help propagate a wide-spread understanding of TK1.

1 Introduction

Cancer is a complex disease and remains the second leading cause of death overall (Siegel et al., 2013). Our knowledge of the biochemical pathways that contribute to malignancy is ever increasing. As we understand more cancer biology, we also identify relevant clinical indicators of malignancy called biomarkers. One class of biomarkers of particular note have been proliferation markers including thymidine kinase 1 (TK1), Ki-67, and proliferating cell nuclear antigen (PCNA). Ki-67 is the most prominent and widely used proliferation marker, although its relevance is limited to immunohistochemical assessment (Dowsett et al., 2011). However, recent evidence indicates that TK1may be a more accurate proliferation marker than either Ki-67 or PCNA in non-small cell, breast, and colorectal carcinomas (Q. He et al., 2004; Y. Mao et al., 2005; Y. Mao et al., 2002; J. Wu et al., 2000). In addition to TK1's potential as a histological biomarker, TK1 is useful as a diagnostic and prognostic serological biomarker. In recent years, TK1 has also been used as an imaging tool in positron emission tomography (PET). The diversity of TK1's clinical applications and its apparent advantage over other proliferation markers warrants a closer look into its biology and clinical relevance. In fact, experts have estimated that "in the next 5-10 years [2013-2018] it may be presumed that thymidine kinase will become a part of the recommended procedures for follow-up in most haematological malignancies and in solid tumors" (Ondrej Topolcan & Holubec, 2008). As a result, this review seeks to highlight these recent advances which have propelled TK1 forward into clinical view.

2 Biology of TK1

2.1 TK1 and TK2 Isoforms

There are two isoenzymes of thymidine kinase: TK1 and TK2. Initially TK1 was identified as TK-fetal while TK2 was identified as TK-adult due to their prevalence in fetal or adult tissue, respectively (Madec et al.,

1988; Munch-Petersen, 1990). TK1 (TK-fetal) was overexpressed in fetal tissue as well as several types of tumors, including breast, colon, lung, and a variety of haematological malignancies (Catalano et al., 1989; De Blasio et al., 1990; Madec et al., 1988; Munch-Petersen, 1990; Musto et al., 1989; Weber et al., 1990). TK2 (TK-adult) was found at stable levels in adult tissues and did not fluctuate with the cell cycle (Q. He et al., 1991). This is primarily because TK2 is directed to the mitochondria by a 40 amino acid leader sequence, and is responsible for maintaining dTTP pools in the mitochondria, independent of the cell cycle (Saada et al., 2001; L. Wang & Eriksson, 2000). Apart from their basic function of maintaining dTTP pools, TK1 and TK2 vary considerably in their basic biology and their relationship to disease. Since TK2 does not play a prominent role in malignancy and is thoroughly reviewed elsewhere, the remainder of this review will focus on TK1 (Munch-Petersen, 2010; Priego et al., 2012).

2.2 TK1 Function

During DNA synthesis, nucleotides are either synthesized de novo or through the salvage pathway where they are recycled from intracellular and extracellular sources. TK1 is one of two major salvage pathway kinases responsible for maintaining the cellular nucleotide pool (Reichard, 1988). TK1 is primarily responsible for the phosphorylation of deoxythymidine (dT). Its product, dTMP, is then subsequently phosphorylated and incorporated into the DNA as dTTP (Reichard, 1988). Expectedly, dTTP helps to regulate this process as it inhibits TK1, the rate-limiting step of this process (Q. He et al., 2002; Munch-Petersen, 2009). Deoxycytidine kinase (dCK) is another salvage pathway kinase which primarily phosphorylates deoxycytidine in addition to deoxyadenosine and deoxyguanosine (Bohman & Eriksson, 1988; Reichard, 1988; Sarup & Fridland, 1987). In 2012, Austin et al. demonstrated that TK1 can help counteract dCK inactivation and restore dCTP levels, illustrating the complex and possibly redundant role of TK1 in maintaining the nucleotide pool balance (Austin et al., 2012). Although ATP and dT are the primary substrates of TK1, several thymidine analogs such as 3'-azido-2',3'dideoxythymidine (AZT), 3'-fluoro-2',3'dideoxythymidine (FLT) have also been utilized as TK1 substrates in clinical applications. One major advantage of AZT and FLT is the lack of confounding phosphorylation by TK2. Munch-Petersen et al. demonstrated that TK2 does not phosphorylate FLT and has a very poor capacity to phosphorylate AZT compared to its typical dT substrate (Eriksson et al., 1991; Munch-Petersen et al., 1991). The clinical applications of these substrates will be discussed in more depth in 3.1 TK1 Methods of Detection and 3.7 TK1 Imaging-Positron Emission Tomography (PET).

Under normal proliferating conditions, TK1 is regulated by the cell cycle (Kauffman & Kelly, 1991). TK1 levels are very low or barely detectable during G_1 phase and begin to increase during late G_1 phase (N. Wang et al., 2001). TK1 levels peak during S phase at concentrations near 200 nM, at least 10-fold higher than levels during G_1 phase (Kauffman & Kelly, 1991; Munch-Petersen et al., 1993; Mutahir et al., 2013; Sherley & Kelly, 1988). Interestingly, Sherley et al. reported that under normal conditions, TK1 mRNA only increased 3-fold or less, compared to the 15-fold increase in protein levels, during the cell cycle (Sherley & Kelly, 1988). They also determined that the rate of [^{35}S] incorporation during S phase was 12-fold more efficient than during the G_1 phase (Sherley & Kelly, 1988). Indicating that the rapid increase in TK1 levels during S phase was a result of an increase in the efficiency of TK1 translation, rather than an increase in transcription. This finding is particularly interesting in light of a study by Chou et al. in which a 5'-untranslated region (5'UTR) allowed translation of TK1 mRNA to be cap-independent (Chou & Chang, 2001). Munch-Peterson et al. has since demonstrated that this rapid increase in TK1 is also a result of conversion from an inactive dimeric to the active tetrameric TK1 form (Munch-Petersen, 2010). On the other hand, Kauffman et al. reported that the rapid decrease in TK1 levels during the G_2 phase is primarily a result c-terminal dependent degradation (Kauffman & Kelly, 1991).

Closely associated with TK1's role in maintaining nucleotide pool balance throughout the cell cycle, is its role in DNA repair. Several studies confirm that TK1 levels increase as a result of DNA damage, especially following irradiation or chemotherapy (Y. L. Chen et al., 2010; Haveman et al., 2006; Kreder et al.,

2002). In 2010, Chen et al. further characterized the connection between TK1 and DNA damage by showing $p53^{-/-}$ tumor cells increased TK1 levels in response to DNA damage while p53 wildtype tumor cells did not (Y. L. Chen et al., 2010). This connection between TK1 and p53 has been corroborated in other studies which report normal p53 function is required to maintain cell cycle dependent regulation of TK1, and upon p53 loss, there is a compensatory increase in TK1 (Radivoyevitch et al., 2012; Schwartz et al., 2004). Closer analysis of this connection revealed that the increase in TK1 levels following DNA damage is dependent on p21 (Y. L. Chen et al., 2010). In fact, Huang et al. showed that the c-terminal domain of p21 interacts with TK1 and overexpression of TK1 prevents p21-dependent growth suppression (D. Y. Huang & Chang, 2001). These results challenged the traditional role of TK1 in tumor cells. For example, Chen et al. determined that TK1 knockdown did not affect the growth of tumor cells, even though the levels of dTTP significantly decreased ($p<0.01$) (Y. L. Chen et al., 2010). Their results support the conclusion that the primary role of TK1 in tumor cells is DNA repair rather than to provide sufficient dTTP levels for replication and growth. This conclusion is supported by several other studies which have shown that functional TK1, resulting in increased dTTP levels, is essential for DNA repair in tumor cells following treatment with an alkylating agent or radiation (al-Nabulsi et al., 1994; Jeong et al., 2004; McKenna et al., 1985; Wakazono et al., 1996). In this way, TK1 operates as a mechanism of resistance which promotes tumor cell survival.

Another surprising and obscure function of TK1 is its possible connection to the immune system. In an effort to understand the biological significance of TK1, Dobrovolsky et al. created TK1 knockout ($TK^{-/-}$) mice (Dobrovolsky et al., 2003). These mice exhibited kidney abnormalities including sclerosis of kidney glomeruli, serous secretion by the salivary gland instead of mucous secretion, a significant decrease in splenic lymphocytes, abnormal lymphoid structure of the spleen, and occasional inflammation of the arteries (Dobrovolsky et al., 2003). Given TK1's connection to proliferation, the cells most likely to be affected in TK1 knockout mice would be rapidly dividing cells or fetal cells. Interestingly, this was not the case, as the most pronounced changes were found in the kidney and salivary glands (Dobrovolsky et al., 2003). The kidney abnormalities coincide with a report by Zaharevitz et al. indicating that mouse kidney cells preferentially rely on the nucleotide salvage pathway over de novo synthesis (Zaharevitz et al., 1992). Similarly, Luo et al. reported that TK1 activity was 3-fold higher in normal human kidney cells compared to renal cell carcinoma (Luo et al., 2009; Luo et al., 2010). To date, this is the only human tissue in which TK1 is overexpressed in normal tissue compared to malignant tissue. Unfortunately, the mechanisms behind the TK1 knockout mice's kidney and salivary gland abnormalities are not understood, nor are the kidney's preferential use of the salvage pathway. On the other hand, the remaining phenotypic changes of the TK1 knockout mice indicate an attenuated or dysfunctional immune system (Dobrovolsky et al., 2003). In particular, the cause of the significant decrease in splenic lymphocytes is not known but could be a result of either failure to activate or improper maturation (Dobrovolsky et al., 2003). There is recent evidence that TK1 is overexpressed during hematopoiesis in normal human bone marrow indicating a potential role of TK1 in the maturation of lymphocytes (Alegre et al., 2013). TK1 may also play a role in autoimmunological diseases, especially of the thyroid (Karbownik et al., 2003; Schwartz et al., 2004). In 2003, Karbownik et al. reported TK1 expression was 2-fold higher in leukocytes from patients with Hashimoto's and Graves' disease compared to healthy controls (Karbownik et al., 2003). Although the connection between TK1 and the immune system is largely unexplored, it seems clear that it plays a significant role in maintaining normal immune function.

Overall, the biochemical role of TK1 is clear. In normal cells, TK1 is responsible for maintaining the dTTP nucleotide pool in a cell cycle-dependent manner. Additionally, TK1 plays an invaluable role in DNA repair and survival of tumor cells following DNA damage. The biological significance of TK1 is less understood and somewhat puzzling. Normal TK1 function is essential for proper development and function of the kidney and salivary gland although these mechanisms are not understood (Dobrovolsky et al., 2003). TK1 also appears to be necessary for the normal function of the immune system and may play a role in its

deregulation. Another unexplored and puzzling function of TK1 is its role in the circulatory system of cancer patients. TK1 is well-known for its elevation in the serum of cancer patients, which will be discussed in detail later. However, the mechanism by which TK1 enters the serum and its function in the serum has been largely unexplored. Perhaps, its function in the serum is connected to regulating the immune system. Further analyses are needed to understand this connection and its significance.

2.3 TK1 Structure

Human TK1 (hTK1) as a monomer, in its most basic structure, is 234 amino acids in length with a molecular weight of 25.5 kDa (Bradshaw & Deininger, 1984). TK1 adopts a variety of oligomeric forms although it is most commonly found as a dimer or tetramer, approximately 53 kDa and 100 kDa respectively (Munch-Petersen, 2009). In 1993, Munch-Petersen reported that the TK1 dimer was the low-efficiency form of the enzyme with a high Km (15uM). On the other hand, the TK1 tetramer was a high-efficiency form with a low Km (0.7uM) and was reported to have 30-fold increased efficiency compared to the dimer in catalyzing its phosphoryl transfer reaction (Munch-Petersen, 2009).

The crystallization of TK1 indicates that the tetrameric form is composed of a dimer of dimers (Segura-Pena, Lutz, et al., 2007). As such, there are two distinct monomer-monomer interfaces labeled strong and weak. The weak interface is primarily stabilized indirectly by ATP, the donor molecule, while the strong interface is stabilized directly through many polar interactions (Segura-Pena, Lutz, et al., 2007). Each monomeric subunit consists of an α/β-domain which is most similar to DNA binding proteins including RecA (Welin et al., 2004). As TK1 catalyzes the conversion of dT to dTMP, the overall structure remains unaltered although Welin et al. reported two regions which do change conformation, the P loop and Lasso loop (Segura-Pena, Lichter, et al., 2007; Welin et al., 2004). These conformational changes occur independently and are only dependent on their respective substrates (Segura-Pena, Lichter, et al., 2007). The P loop or flexible loop consists of residues Gly26-Ser33 in hTK1 and is where the phosphate donor, typically ATP, is held (Birringer et al., 2005; Welin et al., 2004). When ATP binds, this P loop becomes rigid and forms an ordered β-hairpin structure (Segura-Pena, Lichter, et al., 2007). An essential element of the P loop and ATP binding is stabilization by a magnesium ion (Segura-Pena, Lutz, et al., 2007). This magnesium ion is surrounded by two phosphate groups of ATP, three water molecules, and a threonine residue (Segura-Pena, Lutz, et al., 2007; Welin et al., 2004).

On the other hand, the lasso loop consists of residues Leu166-Lys180 and is where the phosphate acceptor molecule, typically dT, is found (Welin et al., 2004). Similar to the P loop, the binding of dT to the lasso loop is closely associated with a metal ion, zinc (Birringer et al., 2005). This zinc-binding motif is located between residues Thr150-Lys191, and is held in place by four cysteines at residues 153, 156, 185, and 188 (Birringer et al., 2005). This situates zinc within 20 Å of the active site, highlighting its crucial role in TK1's phosphoryl transfer reaction (Welin et al., 2004). The pivotal role of zinc is confirmed by Ishikawa et al. who reported that TK1 expression in rats fed a low-zinc diet was reduced by 50% and the percentage of cells in S phase was significantly reduced compared to controls (Ishikawa et al., 2008). The actual phosphorylation reaction of TK1 requires coordination from several different residues. The lasso loop stabilizes dT in a hydrophobic pocket by Met28, Phe101, and Leu24, while the C5 of dT makes contact with Thr163 (Birringer et al., 2005). Glu98 acts as a base which accepts a proton from the 5'OH of dT while Lys32 of the P loop transfers a phosphate group to this 5'OH of dT (Birringer et al., 2005; Segura-Pena, Lutz, et al., 2007). The transitional state during the conversion of dT to dTMP is stabilized by Arg60 (Welin et al., 2004). The remarkable and unique component of TK1's reaction is that the backbone of the protein is involved in this reaction, rather than the side chains (Birringer et al., 2005). This enables hTK1 to limit its substrates to ensure greater specificity unlike the herpes simplex virus TK which has a broad range of substrates and primarily uses side chains to catalyze its reaction (Birringer et al., 2005).

In addition to inducing a conformational change at the monomeric level, ATP binding induces reversible TK1 tetramerization (Munch-Petersen et al., 1993). In the presence of ATP, TK1 switches from a dimer to a tetramer (Kuroiwa et al., 2000; Munch-Petersen et al., 1993). In 2009, Munch-Petersen determined that this TK1 regulatory switch between the dimer and tetramer could occur with ATP, UTP, GTP or CTP indicating that the sugar and base were not involved in tetramerization, rather only the phosphate group was essential (Munch-Petersen, 2009). Although any of these nucleotides were sufficient to induce tetramerization, only ATP could act as a phosphate donor, indicating that ATP-dependent tetramerization and phosphorylation of dT are independent events (Munch-Petersen, 2009). In 2006, Zhu et al. reported that deletion of the c-terminal of TK1 did not effect this ATP-dependent tetramerization (Zhu et al., 2006). On the other hand, Li et al. determined that serine-13 phosphorylation of TK1 disrupted ATP-induced tetramerization, (C. L. Li et al., 2004). Additionally, Mutahir et al. demonstrated how the weak dimer interface is involved in this ATP-dependent regulatory switch. For example, they reported that this interface was composed of two antiparallel α1 helices in which there was only 7.5 Å separating the two dimers at the residues Phe29 and Ile45 (Mutahir et al., 2013). A study by Segura-Pena et al. supported this conclusion in that two additional cysteine residues within the α1 helix, at the weak dimer interface, locked TK1 in the tetramer form (Segura-Pena, Lichter, et al., 2007). In addition to causing tertiary structural changes, the binding of ATP can also induce the transformation of a closed, inactive tetramer to an open, catalytically active tetramer (Segura-Pena, Lichter, et al., 2007). Sengura-Pena et al. reported that during this transformation, the weak dimer interface expands by almost 10% and rotates by 11 degrees (Segura-Pena, Lichter, et al., 2007).

Interestingly, ATP is not the only mechanism by which this regulatory switch can be activated. In 2009, Munch-Petersen reported that the concentration of TK1 can also induce a dimer to tetramer switch, at concentrations higher than 0.2 mg/ml, regardless of ATP (Munch-Petersen, 2009). This confirms results by Birringer et al. who demonstrated that recombinant TK1 was exclusively found in the tetramer at concentrations ranging from 0.4-20 mg/ml (Birringer et al., 2006). Munch-Petersen surprisingly showed TK1 remains as a tetramer at low concentrations (6μg/ml) when analyzed immediately but when the same sample was stored diluted for more than 2 weeks before analysis, TK1 existed as a dimer (Munch-Petersen, 2009). This illustrated that the dimer to tetramer switch is a very slow process which can help explain some discrepancies among other studies.

The dimer to tetramer switch of TK1 does not exist in all organisms and its study among various organisms hints at the structural evolution of TK1 (Mutahir et al., 2013). In a study of the oligomeric structure of TK1 among ten organisms, Mutahir et al. determined that TK1 was only found as a dimer in bacteria, plant, and *Dictyostelium* while all vertebrates and *C. elegans* had tetrameric TK1. Therefore, they deduced that the origin of the TK1 tetramer occurred after the split between the animal and *Dictyostelium* lineages (Mutahir et al., 2013). Furthermore, the regulatory switch from the TK1 dimer to tetramer did not exist in *C. elegans* and *Danio rerio* (zebrafish) and was only fully functional in birds and mammals (Mutahir et al., 2013). This indicated that although the TK1 tetramer was present in all vertebrates, the TK1 regulatory switch from the dimer to tetramer originated with the warm-blooded vertebrate lineage (Mutahir et al., 2013). Further work, including the testing of TK1 from more organisms, is still needed to understand the intricate mechanisms associated with the evolution of TK1's activities.

One intriguing and challenging hurdle in TK1's journey from bench-to-bedside has been its unique structure in malignancy. Initially, Karlstrom et al. reported that the majority of serum TK1 from leukemia patients had a molecular weight of approximately 730 kDa with only a small percentage at 58 kDa (Karlstrom, 1990). On the other hand, only the 58 kDa form could be found in proliferating HeLa cells (Karlstrom, 1990). Since then, Sharif et al. reported this high molecular weight form could be found in CEM cells, although the majority of TK1 was found in the 40-100 kDa fraction (Sharif, Kiran Kumar, et al., 2012). They also confirmed earlier reports as they found that 90% of the TK1 in human leukemia serum was in this same high

molecular weight form, 300-720 kDa (Sharif, Kiran Kumar, et al., 2012). This high molecular weight form has made the detection of TK1 protein levels problematic for two major reasons. First, TK1 activity and protein levels do not correlate unless reducing agents are used, which also partially reduce immunoglobulins (Q. M. He et al., 2005; Sharif, Kiran Kumar, et al., 2012). Additionally, recombinant TK1 does not reflect the same quaternary structure as serum TK1, making it difficult to use an appropriate TK1 positive control for immunoassays. Sharif et al. suggested that methionine and tyrosine residues of TK1, in addition to 7 of TK1's 11 cysteine residues, may contribute to the high molecular weight form of serum TK1. This conclusion was supported as they demonstrated that human serum TK1was found in oligomeric structures irrespective of reducing agents (Sharif, Kiran Kumar, et al., 2012). It is also unclear as to whether this TK1 high molecular weight aggregate is a homo- or hetero-oligomer, indicating the possible existence of a protein binding partner.

Overall it can be seen that the structure of TK1 influences not only its function, but also its regulation and detection. A thorough understanding of the secondary structures and residues involved in TK1's phosphoryl transfer reaction enable us to design clinically relevant substrates such as AZT and FLT. TK1's structure can also help us design TK1 inhibitors which may re-sensitize otherwise resistant tumors. Additionally, a sound understanding of the structure of TK1 in malignancy will ultimately lead us to develop better methods of detection which could be used in the clinic. Finally, the structure of TK1 is particularly informative and essential in understanding the regulation of TK1 since post-translational modifications play a major role in its mechanism of control.

2.4 TK1 Mechanisms of control

TK1 is tightly regulated by the cell cycle. TK1 is found at very low levels in G_1 phase, peaks during S phase, and is degraded during late G_2/M phase (Kauffman & Kelly, 1991; Sherley & Kelly, 1988; N. Wang et al., 2001). Phosphorylation is one common cell cycle mechanism which targets proteins for Skp, Cullin, F-box containing complex (SCF)-mediated degradation (Harper, 2002). Initially, Chang et al. reported that TK1 was heavily phosphorylated in rapidly dividing cells and partially hypo-phosphorylated in M phase-arrested cells, which suggested phosphorylation may play a role in TK1 regulation (Chang et al., 1994; Q. M. He et al., 1996; Lin et al., 2003). Additionally, Ke et al. demonstrated that phosphorylation of serine-13 targets TK1 for SCF-mediated degradation in yeast (Ke et al., 2003). However, Ke et al. later determined that the APC/C-Cdh1 pathway is responsible for TK1 degradation in mammalian cells, not the SCF complex (Ke & Chang, 2004). This indicates that the cell cycle-dependent phosphorylation does not play a role in TK1 degradation (Ke & Chang, 2004). This APC/C-mediated degradation of TK1 explains the very low levels of TK1 at the beginning of the cell cycle, but it does not explain the rapid increase proceeding S phase. Munch-Petersen et al. has demonstrated that this rapid increase is mediated in part by the TK1 regulatory switch from an inactive dimer to an active tetramer (Munch-Petersen, 2009; Munch-Petersen et al., 1995; Munch-Petersen et al., 1993). During G_0 and G_1 phases, TK1 exists as a dimer with estimated concentrations at 0.03-0.09 µg/ml, much lower than the concentration which induces a dimer to tetramer switch (Munch-Petersen et al., 1995; Munch-Petersen et al., 1993). On the other hand, TK1 exists as a tetramer during S phase with estimated concentrations at 4-6 µg/ml (160-240 nM) (Munch-Petersen et al., 1995; Munch-Petersen et al., 1993). This transition is likely regulated by TK1 concentration during G_1 phase (since the concentration is so low), and ATP-dependent tetramerization is responsible for the increased TK1 activity during S phase (Munch-Petersen et al., 1995). Interestingly, phosphorylation in rapidly dividing cells appears to play a role in this dimer to tetramer transition as well, rather than as a target for degradation (C. L. Li et al., 2004). Li et al. demonstrated that serine-13 phosphorylation disrupts ATP-dependent tetramerization, and as a result, TK1 is preferentially found as an inactive dimer (C. L. Li et al., 2004). Since cdc2 kinase phosphorylates serine-13 of TK1 during G_2/M phase, phosphorylation helps to regulate the tetramer to dimer switch and thus decrease TK1 activity following S phase (Chang et al., 1998; Chang et al., 1994).

Protein degradation plays an important role in maintaining appropriate levels of TK1 throughout the cell cycle, as briefly discussed previously. Closely associated with protein degradation is the half-life of TK1. Zhu et al. reported that a 44 amino acid deletion of TK1's c-terminus increased its half-life to 500 minutes, compared to 83 minutes for wild-type TK1 (Zhu et al., 2006). This increased stability illustrated the crucial role of the c-terminus in TK1degradation. Similarly, Demeter et al. reported the half-life of TK1 from normal ovarian epithelial cells was 82 minutes compared to 36 minutes for that of malignant ovarian cells (Demeter et al., 2001). Posch et al. showed that the unstable, decreased half-life (<60 minutes) of TK1 can also be a result of mutation of the binding sites for ATP or dT (Posch et al., 2000). Although this may explain the decreased half-life in malignancy, these mutant TK1s did not increase with proliferation (Posch et al., 2000). Further work is needed to understand the mechanism by which TK1 activity is increased in malignancy since it appears to be unstable in cancer cells compared to that in wildtype cells.

Under normal conditions, TK1 is polyubiquitinylated during the G_2/M phase which targets it for degradation by the APC/C pathway (Ke & Chang, 2004). Additionally, Ke et al. demonstrated that Cdh1 mutant inhibited TK1 degradation whereas a Cdc20 mutant had no significant effect on TK1 degradation, indicating that TK1 is degraded by the APC/C-Cdh1 complex (Ke & Chang, 2004). Cdh1 is the rate-limiting step for this degradation, as it binds to the KEN box (residues 203-205 of the c-terminal end of TK1) (Ke & Chang, 2004). This degradation process does not occur when dT is bound to TK1 (Ke et al., 2007). In addition to acting as a control mechanism for degradation, dT-bound TK1 also reverses Cdh1-mediated expression of TK1(Ke et al., 2007).

In addition to post-translational modifications such as ubiquitination or phosphorylation, TK1 is also subject to transcriptional regulation. The promoter region of TK1is suppressed by a G-quadruplex motif located between -13 and +8 relative to the transcription start site (Basundra et al., 2010). Although this region controls TK1 expression generally, TK1's promoter also has regions responsible for cell cycle-dependent TK1 expression. For instance, initially the promoter region of TK1 (-441 to -63 relative to the transcriptional start site) was identified as the region responsible for cell cycle regulation (Y. K. Kim et al., 1988). Subsequent analysis by Kim et al. determined that the minimum fragment which conferred cell cycle regulation was a 70 bp region between -133 and -64 which was named the cell cycle regulatory unit (CCRU) (Y. K. Kim & Lee, 1991). Within this region, an inverted CCAAT sequence and one G-C rich motif were found between -84 and -64 (Y. K. Kim & Lee, 1992). Kim et al. determined that without this 20 bp region, -84 to -64, the level of transcription dropped to barely detectable levels, indicating that this region acts as an enhancer element (Y. K. Kim & Lee, 1992). Therefore, even though the actual cell cycle control element is found between -133 and -84, the enhancer element from -84 to -64 is also required for proper expression (Y. K. Kim & Lee, 1992). Yi binding factor is known to regulate cell cycle-dependent TK1 expression in mice by binding to the Yi consensus sequence during late G_1/S phase (Dou et al., 1991). Similarly, a Yi-related sequence was identified in the human TK1 promoter between -109 and -84, which is important for cell cycle-dependent TK1 expression (Y. K. Kim & Lee, 1992). Li et al. also determined that cyclin A and $p33^{cdk2}$ complexes were constitutively associated with this site, -109 to -84 (E. C. Kim et al., 1996; L. J. Li et al., 1993). Additionally the binding activity of the cyclin A/p107 complex was increased throughout the S phase and correlated to an increase in TK1 mRNA levels (L. J. Li et al., 1993). Another protein which is associated with cell cycle-dependent TK1 expression is CCAAT binding protein for TK gene (CBP/tk) (Pang & Chen, 1993). This protein binds to the CCAAT sequence located from -91 to -64 of the TK1 promoter, which is known to contribute to promoter strength (Good et al., 1995; Lipson et al., 1995; X. Mao et al., 1995; Pang & Chen, 1993). NF-Y binding to the TK1 promoter is responsible for recruiting CBP/tk to the CCAAT sequence and Sp1 to the region -118 to -113 (Chang et al., 1999; Chang & Liu, 1994). The transcription and post-translational control of TK1 play an important role in maintaining normal TK1 function in a cell. It is through the deregulation of this process, that we begin to see its unregulated elevation in malignancy.

3 TK1 in Malignancy

TK1 has diagnostic, prognostic and therapeutic potential in malignancy. This is true for both TK1 found in the serum (sTK1) as well as TK1 expressed in tumor tissue. An understanding of TK1's various methods of detection is critical to understanding sTK1 diagnostic and prognostic potential.

3.1 TK1 Methods of Detection

The unique structure of sTK1, found in its high molecular weight form (300-720 kDa), has been a major challenge in developing a clinically relevant methods of detection. Furthermore, He et al. reported a puzzling observation in which malignant serum, high in TK1, diluted with normal serum reduced both TK1 concentration and activity more than expected (Q. M. He et al., 2005). They also reported this same finding when malignant serum was diluted with BSA, indicating there is a factor in normal human and calf serum that destabilizes TK1 (Q. M. He et al., 2005). Another puzzling and unique aspect of sTK1 is its long half-life. The half-life of TK1 is one hour in mouse EAT cells, four hours in HeLa cells, and a surprising 30 days in serum (Q. He et al., 1991; Q. He et al., 2000; Hengstschlager et al., 1994). The 30 day half-life of sTK1 has been confirmed for both breast cancer and gastric cancer (Q. He et al., 2000; Zou et al., 2002). Clearly, the environment of sTK1 contributes to its unique structure and properties in ways that can't always be predicted by studying TK1 in cancer cells alone.

Consequently, the traditional method of quantifying TK1 is by measuring its activity using a radioassay. For this assay, [^{3}H]-dThd is phosphorylated using either ATP or CTP (K. L. O'Neill et al., 1986). [^{3}H]-dTMP then absorbs to ion exchange DE81 filter paper, is washed and radioactivity is measured. TK1 only utilizes ATP while TK2 utilizes both ATP and CTP, so the relative contribution of TK1 can be determined by using both ATP and CTP separately (Bristow et al., 1988). In addition to these substrates, the reaction mixture varies slightly in its additives such as $MgCl_2$ and/or KCl, but a reducing agent, to break disulfide bonds, is crucial for the assay to ensure reliable, consistent results (K. L. O'Neill et al., 1986; Sharif, von Euler, et al., 2012a). In 2012, Sharif et al. optimized this traditional radioassay specifically for human sTK1 (Sharif, von Euler, et al., 2012a). They reported the area under the curve (AUC) was 0.94 with a sensitivity and specificity of 0.89 and 0.74 respectively (Sharif, von Euler, et al., 2012a). The traditional TK radioassay was commercialized and sold as a kit. Instead of [^{3}H]-dThd, the kit utilizes [^{125}I]-deoxyuridine (H. P. Von Euler et al., 2009). This same technology, slightly modified, is sold as either the radio-receptor analysis (RRA) kit (Immunotech, Czech Republic) or more commonly as the TK Prolifen assay or TK-REA (DiaSorin AB, Sweden) (Span et al., 2000; H. P. Von Euler et al., 2009; Votava et al., 2007). Unfortunately, Svobodova et al. reported that sTK1 activity levels, using the RRA test, were significantly elevated in relatively few cases of 1087 cancer patients compared to healthy controls (Svobodova et al., 2007). Despite the low cost of this test ($4/sample), these results should be interpreted with caution since they do not always reflect typical trends of TK1 in malignancy (Votava et al., 2007). Today, the TK-REA kit is commonly used as a method of comparison for new ways to quantify TK1. For example, the TK-REA has been used in side-by-side comparisons with new assays especially the Liason, described later (Ohrvik et al., 2004). On the other hand, the traditional, non-commercialized radioassay has been the standard method to determine TK1 activity since the late 1980's, and has also been used in several side-by-side comparisons (Luo et al., 2009; McKenna et al., 1988; K. O'Neill et al., 1987; K. L. O'Neill et al., 2001; K. L. O'Neill, Hoper, et al., 1992; K. L. O'Neill, McKelvey, et al., 1992; Robertson et al., 1990; Thomas et al., 1995). Unfortunately, there are several disadvantages of the traditional radioassay and TK-REA. For example, these tests are radioisotope-based, relatively expensive, time consuming, require specialized training and facilities, are relatively inconsistent, and have relatively low sensitivity (F. Zhang et al., 2001).

Fortunately, in the late 1990's and early 2000's, anti-TK1 antibodies prompted the development of immunoassays which could be clinically applicable. TK1-specific antibodies proved problematic since TK1 is highly conserved among mammals (89-97% among humans, mice, and rabbits). Therefore, currently the only clinically robust antibodies are ones which bind the c-terminal fragment or the 24-amino acid active site called the lasso loop of TK1 (H. von Euler & Eriksson, 2011). In 2001, Zhang et al. reported a TK1-specific monoclonal antibody which blocks the active site of TK1. This was later used to develop an enzyme-linked immunosorbent assay (ELISA) (F. Zhang et al., 2001). This ELISA showed a strong correlation with the traditional radioassay and gave similar results without many of the disadvantages associated with the radioassay. The majority of clinical biomarkers today utilize a similar technique called a Sandwich ELISA. The Sandwich ELISA is highly specific and sensitive since it relies on two TK1-specific antibodies. In 2009, Carlsson et al. developed a Sandwich ELISA with AUC values of 0.56, 0.73, and 0.64 for postoperative, disease recurrence, and chemotherapy treated patients respectively (Carlsson et al., 2009). AUC values for useful biomarkers are typically closer to 0.86 as is the case with CA 72-4, a biomarker for gastric cancer (Fernandez-Fernandez et al., 1996). Unfortunately, this TK1 Sandwich ELISA has AUC values near 0.5, the value indicating the assay has no predictive power. Furthermore, the sensitivity and specificity of this assay were surprisingly low, indicating that it is not a clinically useful TK1 immunoassay (Carlsson et al., 2009). As a result, no further studies have been conducted with this assay. There is still a need for a robust Sandwich ELISA which can be quickly integrated into the clinic to aid in diagnosis and prognosis of malignancy.

In order to circumvent the highly conserved nature of TK1 and make robust, high-affinity TK1 antibodies, Wu et al. developed a chicken polyclonal IgY antibody specific to the c-terminus of hTK1 (C. Wu et al., 2003). This IgY TK1 antibody was used to develop a dot blot to quantify TK1 protein levels (TK1p) instead of TK1 activity (TK1a) (C. Wu et al., 2003). This dot blot is highly sensitivity with the lowest detectable concentration of TK1p at 33.3 pg/ml (C. Wu et al., 2003). In the past 10 years, this dot blot has been used in 14 studies including over 3,500 cancer patients and almost 70,000 healthy individuals (Y. Chen et al., 2010; Chen et al., 2011; Chen et al., 2008; E. He et al., 2010; Q. M. He et al., 2005; Kameyama et al., 2011; H. X. Li et al., 2005; Z. Li et al., 2010; Liu et al., 2011; Pan et al., 2010; C. Wu et al., 2003; X. H. Xu et al., 2008; J. Zhang et al., 2006). In 2011, Chen et al reported the AUC of the dot blot for a healthy screen of 35,365 individuals was 0.96 (Chen et al., 2011). They also reported that the specificity and sensitivity were 0.99 and 0.78 respectively, while only 0.8% of healthy city-dwellers had elevated sTK1 levels, which typically corresponded to pre-malignant conditions (Chen et al., 2011). As of 2010, this dot blot has been commercialized, and the kit was approved by the Supervision Authority for Food and Medicine in China (Pan et al., 2010). This commercialized, approved kit has been one of the biggest factors in propelling TK1 into clinical view during the past few years.

There have been several reports, including use of this commercial dot blot, which have demonstrated that TK1a and TK1p do not correlate (Kristensen et al., 1994; Luo et al., 2009; Sharif, Kiran Kumar, et al., 2012). This has provided the rationale for some to focus on TK1a rather than TK1p. A focus on TK1a also circumvents the challenges associated with developing TK1 antibodies for immunoassays. As a result, Von Euler et al. developed a commercialized competitive ELISA to quantify TK1a using AZTMP antibodies (H. P. Von Euler et al., 2009). This novel assay, currently referred to as the Liaison TK assay, simultaneously exploits the advantages of sensitivity and accuracy associated with an ELISA and negates the controversy regarding protein levels and activity. Although this assay is advantageous in that TK2 does not phosphorylate AZT, yielding no confounding TK2 activity, the Liaison TK assay has some disadvantages (Munch-Petersen et al., 1991). For example, hTK1 is known to phosphorylate dT three times more efficiently than AZT (Eriksson et al., 2002). Similarly, Sharif et al. reported that canine TK1 phosphorylated AZT three times more efficiently than hTK1 (Sharif, Kiran Kumar, et al., 2012). This indicates that the Liason TK assay may be better suited for screening canine malignancies than human malignancies. In fact, many of the studies involving the Liaison TK

assay have investigated canine malignancies (Sharif, von Euler, et al., 2012a; Thamm et al., 2012; H. P. Von Euler et al., 2009). Additionally, Ohrvik et al. demonstrated that the AZTMP antibodies can cross-react with endogenous antibodies in human serum (Ohrvik et al., 2004). In comparison with other TK assays, the Liaison TK assay showed significant linear correlation with the TK REA ($p<0.0001$) (Sharif, von Euler, et al., 2012a; H. P. von Euler et al., 2006; H. P. Von Euler et al., 2009).

The Liaison TK assay, traditional TK assay, TK REA, and TK1 dot blot are among the most popular and well-established methods of detection for TK1. There have been a few other assays which have been developed either for commercial use or research purposes. The DiviTum kit (Biovica/Ronnerbol, Sweden) is another ELISA which measures TK activity, except it uses bromo-deoxyuridine as a substrate (Nisman et al., 2013). DiviTum and Liaison TK assays are very similar although the DiviTum is a manual assay and the Liaison is automated. Nisman et al. demonstrated that both assays were correlated and had efficacy in predicting recurrence preoperatively in breast cancer patients (Nisman et al., 2013). Similarly, the DiviTum assay is efficacious in renal cell carcinoma and non-small cell lung cancer in addition to breast cancer (Korkmaz et al., 2013; Nisman, Yutkin, et al., 2010). Faria et al. measured TK activity in hepatocellular carcinoma patients using liquid chromatography-MS/MS through the phosphorylation of FLT (Faria et al., 2012). Alternatively, Tzeng et al. utilized capillary electrophoresis to separate and quantify dTMP following the traditional TK assay, negating the need for radioisotopes (Tzeng & Hung, 2005). On the other hand, some have developed methods to estimate protein concentrations while still circumventing the challenge of TK1 antibodies. Stalhandske et al. reported a PCR-based real-time assay in which they simultaneously measured both TK1 and dCK levels (Stalhandske et al., 2013). These methods, although practical and valid, have not yet gained widespread popularity. Nevertheless, a thorough understanding of the various methods of TK1 detection, including their limits and strengths, will help shed light on potential discrepancies found as we explore the trends of TK1 in malignancy.

3.2 Serum-TK1 Diagnostic potential

Elevated sTK1in malignancy is a very early event. In 2008, Xu et al. reported 70% of pre-malignant cervical cancer cases were accompanied by elevated sTK1 protein levels (X. H. Xu et al., 2008). Additionally, the mean age of pre-malignant cervical cancer patients was 10 years earlier, compared to cervical cancer patients (X. H. Xu et al., 2008). There have been 3 large Chinese health screens which sought to determine sTK1p levels in healthy adults. In 2008, Chen et al. reported that in a health screen of 11,880 individuals, 0.5% had elevated sTK1p levels, using a previously determined cut off value of 2 pM (Chen et al., 2008). Of the 0.5% with elevated sTK1, 83% reported malignancy-related diseases including benign or hyperplasia tissues (Chen et al., 2008). Similarly, a health screen involving 35,365 individuals revealed that 0.8% of urban-dwelling and 5.8% of oil-field workers had elevated sTK1p levels (Chen et al., 2011). Of the individuals with elevated sTK1p, 8.8% developed new pre-malignancies or showed progression in existing pre-malignancies (Chen et al., 2011). Additionally, elevated sTK1p levels were associated with a 3-5 fold increased risk of developing malignancy within 5-72 months (Chen et al., 2011). This was confirmed in another health screen of 8,135 individuals in which 1.1% had elevated sTK1p levels (S. Huang et al., 2011). Huang et al. reported that of those with elevated sTK1p, one individual developed liver carcinoma within 13 months and five individuals showed malignancy-related disease progression within 19 months (S. Huang et al., 2011). Overall, incidence of elevated sTK1p levels (>2pM) in healthy adults is low and is typically indicative of pre-malignant or malignancy-related diseases. sTK1 levels appear to play a valuable role in early detection of malignancy which may ultimately improve cancer mortality rates.

In addition to early detection, sTK1a and sTK1p levels are significantly elevated in a variety of haematological and solid tumors. Initially, sTK1a was studied primarily in haematological malignancies. Sharif et al. determined that sTK1 activity levels were significantly elevated in both chronic lymphocytic

leukemia (CLL) and myelodysplastic syndrome (MDS), a type of preleukemia (Sharif, von Euler, et al., 2012b; W. Xu et al., 2009). In this study MDS sTK1 levels were the highest among the heamatological malignancy group (Sharif, von Euler, et al., 2012b). This coincides with an earlier study which reported that elevated sTK1a levels were indicative of progression of MDS into acute myeloid leukemia (AML) (Musto et al., 1995). Significantly elevated sTK1a levels compared to healthy controls were also found in pre-treated acute lymphoblastic leukemia (ALL), non-Hodgkin lymphoma, and follicular lymphoma (K. L. O'Neill et al., 2007; Pan et al., 2010; Prochazka et al., 2012). Although sTK1a levels were not initially thought to be a good indicator for solid tumors, we now know elevated sTK1 levels are significantly elevated compared to controls in virtually all solid tumors. This is true for malignant melanoma, systemic breast, preoperable primary breast, gastric, kidney, bladder, non-small cell lung (NSCLC), esophageal, cardiac, cervical, ovarian, colon, rectum, liver, head and neck, thyroid, and brain cancers, among others (Y. Chen et al., 2010; Elfagieh et al., 2012; Fujiwaki et al., 2001; Q. He et al., 2000; Q. M. He et al., 2005; Z. Li et al., 2010; Liu et al., 2011; Nisman, Allweis, et al., 2010; Nisman, Yutkin, et al., 2010; Robertson et al., 1990; Thomas et al., 1995; B. J. Wu et al., 2013; C. Wu et al., 2003; W. Xu et al., 2009; J. Zhang et al., 2006; Zou et al., 2002). Additionally, several studies have investigated whether there is a significant difference between benign and malignant tissue. He et al. reported in a study of 9 types of carcinoma tissue that sTK1 levels of pre-treated malignancy were significantly higher than either benign or noncancerous individuals (Q. M. He et al., 2005). These findings, which indicate there is a significant difference in sTK1 levels in benign and malignant tissue, have been confirmed in several studies, although there is no significant difference in sTK1 levels between benign kidney and renal cell carcinoma (Q. He et al., 2000; Nisman, Yutkin, et al., 2010; X. H. Xu et al., 2008). High sTK1 levels typically indicate a more advanced grade, stage, increased T-values, and increased tumor size (Y. L. Chen et al., 2010; H. X. Li et al., 2005; Z. Li et al., 2010; Nisman, Allweis, et al., 2010). Although some studies have reported that sTK1 is associated with stage but not grade in tumors such as renal cell carcinoma, esophageal, cardiac, and bladder carcinoma (Z. Li et al., 2010; Nisman, Yutkin, et al., 2010; J. Zhang et al., 2006). It is difficult to determine whether or not sTK1 levels in general correlate with grade in each of these carcinoma types, since this information was not always studied. A more thorough analysis of each cancer type is needed to determine if these results are specific to the cancer type or a result of the study's sample population. The overall trends of sTK1 levels and their early elevation among a wide variety of malignancies are clear. Over the years, many cancer biomarkers have been validated and show clinical promise. However, the unique ability of sTK1 to predict risk for malignancy months or even a year before the clinical manifestation of malignancy is an indispensable clinical tool.

3.3 Serum-TK1 Prognostic potential

In many haematological and solid tumors especially those for which sTK1 levels are significantly elevated, sTK1a and sTK1p is a prognostic factor. Responses to chemotherapy and/or surgery are often associated with sTK1 levels. For example, Zhang et al. compared preoperative sTK1p in bladder carcinoma patients with postoperative sTK1 levels at 1 week, 1, 3, and 6 months (J. Zhang et al., 2006). They reported sTK1p levels were 66% lower at 1 week postoperatively, reached normal levels (<2 pM) at 1month, and remained in the normal range until the study ended at 6 months (J. Zhang et al., 2006). Similarly, Li et al. reported in non-metastatic NSCLC patients, 1 month postoperative sTK1p levels decreased significantly by 45%, compared to preoperative sTK1 (H. X. Li et al., 2005). Conversely, they reported that sTK1p levels in metastatic NSCLC patients did not significantly change 1 month postoperative. This same trend was seen with metastatic and non-metastatic breast cancer surgery patients (Q. He et al., 2000). Zou et al. saw the same significant decrease in postoperative sTK1 levels for gastric cancer although only sTK1p, not sTK1a, decreased (Liu et al., 2011; Zou et al., 2002).

Pre-treatment sTK1 levels have been shown to predict which patients are most likely to respond to treatment. Di Raimondo et al. demonstrated that 83% of CLL patients with complete response (CR) or partial response (PR) to fludarabine initially had sTK1a levels < 10 U/L. On the other hand, only 45% of the CLL patients with sTK1a levels ≤ 10 U/L had CR or PR, a significant difference compared to patients above this threshold (Di Raimondo et al., 2001). Alternatively, during chemotherapeutic treatment, sTK1 levels fluctuate depending on a patient's response. Robertson et al. tracked the sTK1a levels of 10 advanced breast cancer patients bimonthly during the first 6 months of their hormone therapy (Robertson et al., 1990). Five patients responded positively to treatment with a resulting decline in their sTK1a levels while five patients progressed while on their treatment and showed increased sTK1a levels. Interestingly, Liu et al. reported no overall significant decrease in sTK1p of gastric patients after 1, 2, or 4 cycles of chemotherapy unless patients were sorted according to their response. Only those patients with either CR, PR or no recurrence had significantly decreased sTK1p levels after cycle 2 of chemotherapy, although the levels began to decline after cycle 1. The patients with either disease progression or recurrence during chemotherapy had increased sTK1p levels (Liu et al., 2011). To determine what happens immediately after treatment, Pan et al. compared pre-treatment sTK1p levels to levels at day 1 and 28 of chemotherapy. They reported a significant increase in sTK1p levels at day 1 followed by a significant decrease correlated with response at day 28, at which time it reached normal sTK1p levels (Pan et al., 2010). This peak in sTK1p during the first day after chemotherapy may be explained by TK1 being released from cancer cells as a result of cell death, which is an indication of effective treatment (Pan et al., 2010). Di Raimondo et al. supported this conclusion as they showed sTK1a levels were correlated with those of beta2-microglobulin and lactate dehydrogenase, indicators of tumor cell turnover (Di Raimondo et al., 2001). Xu et al. similarly reported that sTK1p levels in a variety of malignancies increased by 40-50% during the first month of treatment and subsequently decreased to normal levels (X. H. Xu et al., 2008). The same trends in which sTK1a and sTK1p levels reflect response to treatment have been confirmed in many cancers including lung, esophageal, head and neck, thyroid, leukemia, and colon cancer (Y. Chen et al., 2010; O. Topolcan et al., 2005; Votava et al., 2007).

In addition to cancer monitoring, sTK1 levels are indicative of survival. In CLL patients, high sTK1 levels were associated with a 22% survival rate compared with a 65% survival rate in patients with low sTK1 (Di Raimondo et al., 2001; Fujiwaki et al., 2001; Konoplev et al., 2010). Similarly, in operable breast cancer patients high sTK1 is associated with shorter disease specific survival (DSS), local recurrence free survival (RFS) and distant relapse free interval (Broet et al., 2001; Elfagieh et al., 2012). In renal cell carcinoma and non-Hodgkin lymphoma high sTK1 indicated a lower 5-year RFS (Nisman, Yutkin, et al., 2010; Pan et al., 2010). Similarly, Liu et al. determined that monitoring sTK1 levels during the first 2 months of palliative treatment for advanced gastric cancer was more indicative of progression free survival (PFS) and RFS compared to initial baseline sTK1 (Liu et al., 2011). In advanced breast cancer patients, the 5-year disease free survival (DFS) with high sTK1 was 21%, with a median of 23 months; while patients with low sTK1 had 56% DFS, with a median >30 months (Z. H. Huang et al., 2012). sTK1 was also able to subcategorize nonsmoldering CLL patients at risk for rapid disease progression. Hallek et al. reported nonsmoldering patients with high sTK1a had PFS as expected, 8 months. On the other hand, nonsmoldering patients with low sTK1a had a PFS of 49 months, more typical of smoldering CLL, which indicated that sTK1 was able to identify which nonsmoldering CLL patients were at risk for rapid progression (Hallek et al., 1999).

Early detection of recurrence and elevated risk for recurrence are also associated with increased sTK1 levels. Generally, sTK1 levels are significantly higher, 50-60%, in patients with recurrent tumors compared with primary tumors (Z. Li et al., 2010; K. L. O'Neill et al., 2007; X. H. Xu et al., 2008). Huang et al. estimated that patients were 6-7 times more likely to get recurrence if sTK1p levels were high after neoadjuvant therapy (Z. H. Huang et al., 2012). He et al. also reported that 63% of breast cancer patients who recurred up to 18 months after surgery, had higher sTK1p but not elevated sTK1a (Q. He et al., 2006). In

addition to indicating a risk for recurrence, sTK1 levels begin to rise months before the clinical manifestation of recurrent tumors. For example, Votava et al. reported that sTK1a began to elevate 1 month before a diagnosis of recurrence in childhood leukemia patients (Votava et al., 2007). Svobodova et al. estimated that sTK1a levels increased at least 3 months before recurrence as detected by imaging methods, although the increase could be seen sometimes as early as 6 or 9 months before the clinical manifestation of recurrence (Svobodova et al., 2007). Since recurrent tumors play a major role in cancer mortality, detecting recurrence through sTK1 levels several months earlier could help give clinicians an upper hand towards effective treatment.

Currently, there are nine FDA-approved cancer biomarkers including CEA, CA15-3, and CA19-9 (Rhea & Molinaro, 2011). CA15-3 is a breast cancer biomarker which is known for its poor sensitivity and specificity, but is approved for monitoring breast cancer treatment and recurrence (Rhea & Molinaro, 2011). CA15-3 and sTK1p levels were compared in breast cancer patients preoperatively and 3 months postoperatively (Q. He et al., 2006). sTK1p, but not CA15-3, was significantly increased with recurrence which indicated that sTK1 may be a better marker for breast cancer recurrence than CA15-3 (Q. He et al., 2006). Although CEA is primarily approved for colon cancer monitoring, it is also elevated in breast cancer patients (Chevinsky, 1991). Elfagieh et al. compared CEA, CA15-3, and sTK1 levels for breast cancer prognosis (Elfagieh et al., 2012). They determined that increased levels of CEA, CA15-3, and sTK1 were found in 62%, 70%, and 78% of breast cancer patients respectively. They also reported a combined evaluation of all 3 biomarkers increased the sensitivity to 90%, which indicated that the most accurate diagnosis of breast cancer can be determined using all 3 biomarkers (Elfagieh et al., 2012). In a comparison study of colon cancer, only sTK1a levels, not CEA or CA19-9 levels, changed during chemotherapy treatment (O. Topolcan et al., 2005). Clearly, sTK1 levels provide more clinically relevant prognosis for monitoring cancer patients than several of the FDA-approved biomarkers currently in clinical use. Although sTK1 is also a worthy screening tool, its efficacy in cancer monitoring and prognosis fills a clinical need which may provide the driving force to propel TK1 into clinical use.

3.4 Tumor-TK1 Diagnostic potential

In addition to efficacy as a serum biomarker, TK1 has valuable potential as a means of diagnosis and prognosis in tumor tissue. TK1 expression in tumor tissue (tTK1) has repeatedly been shown to be a more relevant proliferation marker than PCNA. Although both TK1 and PCNA were overexpressed in malignant tissue compared with normal tissue, only TK1 was significantly increased with both grade and stage (Y. Mao et al., 2002; C. Wu et al., 2003; J. Wu et al., 2000). This was true for advanced breast, liver, thyroid, and colon cancer, although PCNA was associated with stage in colon cancer (Y. Mao et al., 2002; C. Wu et al., 2003; J. Wu et al., 2000). Ki67 and BrdU labeling are two other proliferation markers which have been compared with TK1. In 2009, Gasparri reported that TK1 expression during the cell cycle occurs earlier than either Ki67 or BrdU labeling, referred to as an activated G_1 state, which is high in TK1 and low in Ki67 (Gasparri et al., 2009). Virtually all comparison studies reported a strong positive correlation between Ki67 and tTK1 expression in both malignant and pre-malignant conditions such as breast atypical ductal hyperplasia tissue (ADH), NSCLC, and infiltrating ductal breast carcinoma (Brockenbrough et al., 2009; Guan et al., 2009; Q. He et al., 2004). There are slight differences between Ki67 and tTK1 expression. Mao et al. reported a significant difference between tTK1 and Ki67 expression in lung adenocarcinoma tissue but not squamous cell carcinoma. In adenocarcinoma tissue, the significant increase of tTK1 expression resulted mostly from staining of stage 2 and grade 2 tumors (Y. Mao et al., 2005). Since there were also tumors which expressed Ki67 only, a combination of tTK1 and Ki67 was recommended for routine testing (Y. Mao et al., 2005). This recommendation was confirmed by He et al. who reported that the combination of tTK1 and Ki67 expression in breast carcinoma tissue detected the most tumors (Q. He et al., 2004). Zacchetti et al. ranked the

proliferation markers according to their performance in breast tumors in this order PCNA<Ki67<BrdU labeling (Zacchetti et al., 2003). Although this study did not include tTK1, tTK1 is elevated earlier in the cell cycle than Ki67 or BrdU, and can identify tumors which Ki67 misses, indicating that tTK1 may be a more accurate proliferation marker (Guan et al., 2009).

Similar to sTK1 levels, tTK1 overexpression is an early event and can help identify pre-malignancies. Guan et al. demonstrated that tTK1 was positive in 80-90% of ADH, ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC) but only positive in less than 5% of usual ductal hyperplasia (UDH) (Guan et al., 2009). This indicated that ADH, a pre-malignancy, was at increased risk for progression and was significantly higher in tTK1 expression compared to UDH (Guan et al., 2009). The increase of TK1 early in the cell cycle, referred to as an activated G_1 state, is also indicative of the early increase of tTK1 (Gasparri et al., 2009). Although the overexpression of tTK1 in pre-cancerous tissue has been confirmed, Alegre et al. demonstrated that tTK1 was not elevated in prostate hyperplasia tissue (Alegre et al., 2012, 2013). However, since sTK1 is elevated in some cases of prostate hyperplasia, further work is needed to understand the clinical significance of elevated TK1 in prostate hyperplasia (Chen et al., 2011; S. Huang et al., 2011).

It is well established that tTK1 expression is significantly higher, compared to corresponding normal tissue, in breast, liver, thyroid, lung, colon, kidney, esophageal, uterine, prostate, and stomach cancer (Alegre et al., 2013; Y. Mao et al., 2005; Y. Mao et al., 2002; C. Wu et al., 2003; J. Wu et al., 2000). Occasionally, the TK1 antibody used can affect the results of the study. Mao et al. found differences when NSCLC tissue was stained with a c-terminal mouse monoclonal (mAb) and c-terminal chicken anti-TK1 antibody (Y. Mao et al., 2005). Although there was not a significant difference in percentage of tTK1 positive tumors, the distribution of staining varied. The mAb primarily stained stage 2 and grade 2 tumors, with a decrease in tTK1 positive staining in more advanced tumors, while the chicken antibody showed the opposite result with the highest degree of staining in the more advanced tumors (Y. Mao et al., 2005). Although some reports using serum and tumor tissue have indicated that TK1 in kidney cancer is significantly elevated compared to normal tissue, Mizutiani et al. reported tTK1 activity was 4 fold higher in normal kidney tissue compared to renal cell carcinoma (Mizutani et al., 2003). In a subsequent study, they confirmed these findings and to date, this remains the only tissue reportedly higher in normal tissue than corresponding malignant tissue (Luo et al., 2010). These discrepancies remain unclear although a thorough analysis of the differences among the antibodies used could shed light on this controversy. Nevertheless, standardization of scoring and a full characterization of the TK1 antibodies would enable clinicians to make needed comparisons as TK1 transitions from the bench to the bedside.

Recently a derivative of TK1 was also used to stain tumor tissue. XPA-210 is a c-terminal TK1 peptide fragment which includes amino acid 210 (Aufderklamm et al., 2012). Typically tTK1 is a cytoplasmic marker, but XPA-210 expression as detected by a mAb, is located primarily in the nucleus which is more comparable to Ki67 (Gakis et al., 2011). Although there have only been 3 studies involving XPA-210, the trends for prostate and renal cell carcinoma appear to confirm corresponding studies with tTK1 (Aufderklamm et al., 2012; Gakis et al., 2011; Kruck et al., 2012). Regardless of which antibody or peptide fragment of TK1 is used, it remains clear that tTK1 is significantly overexpressed in a variety of tumor tissue, including some cases of pre-malignancy.

3.5 Tumor-TK1 Prognostic potential

tTK1 corresponds with sTK1 in that both have diagnostic and prognostic potential. Just as sTK1 correlated to survival, recurrence, and treatment, similar trends are seen with tTK1. Xu et al. reported that high tTK1 expression was associated with significantly worse 5 year survival in pT1 lung adenocarcinoma patients (Y. Xu et al., 2012). Additionally, Romain et al. reported tTK1 expression in node-negative breast cancer tumors was an independent factor for metastatic free survival and DFS (Romain et al., 2000). Their subsequent study

similarly reported high tTK1 increased the risk of developing distant recurrence and therefore, tTK1 was able to identify which node-negative patients were at a high risk for metastasis (Romain et al., 2001). Aufderklamm et al. similarly reported that higher XPA-210, a fragment of TK1, was associated with shorter time to recurrence and metastasis (Aufderklamm et al., 2012).

In addition to tTK1 expression, the TK1 activity of tumor tissue (tTK1a) is also associated with patient prognosis. Demeter et al. reported the activity of tTK1a was 12-fold higher in ovarian carcinoma, but Mizutani et al. reported only a 2-fold increase for bladder carcinoma relative to normal controls (Demeter et al., 2001; Mizutani et al., 2002). Mizutani et al. also showed that those with low tTK1a had longer RFS (Mizutani et al., 2002). Similarly, O'Neill et al. reported that tTK1a levels in the initial primary breast tumor were associated with recurrence, in that those with recurrence also initially had significantly higher sTK1a levels (K. L. O'Neill, McKelvey, et al., 1992). In a study by Foekens et al. the initial sTK1 activity level also affected the duration of response in advanced breast cancer (Foekens et al., 2001). For tumors with low, intermediate or high tTK1a, the duration of response was 23, 15, and 13 months respectively (Foekens et al., 2001). In renal cell carcinoma, the tTK1a levels are inversely associated with sensitivity to 5-fluorouracil (5FU), which is likely associated with TK1's role in DNA repair, as previously discussed (Mizutani et al., 2003).

Unfortunately there is a need to correlate a patient's tTK1 and sTK1 levels to determine if they are redundant. Both sTK1 and tTK1 generally share the same trends in diagnosis and prognosis. TK1 levels are elevated very early including in pre-malignancy in both serum and tumor tissue. Additionally, increased TK1 in serum and tumor is associated with worse prognosis and disease progression including recurrence. Finally, serum and tumor TK1 appear more accurate and indicative of patient prognosis when compared with other biomarkers including proliferation markers. The efficacy and utility of monitoring TK1 levels both in malignant and healthy individuals remains clear.

3.6 TK1 Therapeutic potential

Although the primary clinical value of TK1 is in its diagnostic and prognostic potential, TK1 also has limited therapeutic potential. TK1 plays an intricate role in DNA repair and maintaining dTTP levels, as discussed previously. Franciullino et al. demonstrated that overexpression of TK1leads to desensitization of tumor cells to 5FU (Fanciullino et al., 2006). Somewhat surprisingly, they also showed that TK1 does not limit the production of 5FU-monophosphate, indicating as previously discussed, that TK1 is involved in DNA repair. Due to the redundant nature of TK1, Di Cresce et al. targeted both TK1 and thymidylate synthase (TS), the salvage and de novo pathways respectively, with siRNA which successfully re-sensitized tumors to 5FU treatment (Di Cresce et al., 2011). A study by Wakazono et al. demonstrated that a decrease in dTTP levels made tumor cells hypersensitive to treatment with alkylating agents (Wakazono et al., 1996). Since TK1 and TS both contribute to dTTP levels, this appears to explain the importance simultaneous knockdown of TK1 and TS to re-sensitize tumors.

TK1 is also used in gene therapy although instead of hTK1, herpes simplex virus TK (HSV-TK) is utilized. A virus-based vector delivers the HSV-TK to gliomas or other tumors in connection with ganciclovir treatment, a harmless prodrug (J. X. Zhang et al., 2010). HSV-TK acts as a suicide gene by cleaving ganciclovir into a toxic compound (J. X. Zhang et al., 2010). This system works for a variety of tumors and has been reviewed thoroughly elsewhere (Oh et al., 2010). Since HSV-TK varies considerably from human TK1 in structure, function and characteristics in malignancy, we will not discuss it further in this review.

3.7 TK1 Imaging: Positron Emission Tomography (PET)

PET imaging is a clinical tool used to help determine tumor metabolism. Flurodeoxyglucose (FDG) is the approved substrate for PET imaging. Unfortunately, FDG-PET is limited in that metabolism is complex and

FDG only assesses one aspect of cellular metabolism (Shields, 2012). In an effort to understand more about tumor proliferation to enable more accurate prognosis, 3'-deoxy-3'-fluorothymidine (FLT) and other substrates were created (Agarwal et al., 2013; Bading & Shields, 2008; Struthers et al., 2010). Katz et al. reported that FLT, but not FDG, predicted response to TRAIL and sorafenib treatment in tumors with functional p53 (Katz et al., 2011).

FLT is phosphorylated by TK1, producing FLT-MP which is then trapped in cells. Unfortunately, FLT-PET is not currently in clinical use. This is most likely because there is tremendous controversy regarding whether FLT uptake into cells correlates with proliferation, measured by TK1 and/or Ki67 levels. Shinomiya et al. recommended that FLT should not be used as a measure of proliferation since FLT phosphorylation did not reflect either tTK1 expression or tTK1 mRNA levels (Shinomiya et al., 2013). Zhang et al. agreed as they reported low FLT uptake with corresponding high TK1 and Ki67 expression in tumor tissue (C. C. Zhang et al., 2012). Several others have agreed that either TK1 or Ki67 expression is not associated with FLT uptake (Benz et al., 2012; Lee et al., 2011; McKinley et al., 2013). Conversely, Brockenbrough et al. reported that FLT uptake was correlated with tTK1 and Ki67 expression but not tTK1a (Brockenbrough et al., 2011). Still others have reported FLT uptake strongly correlated with tTK1a and/or tTK1 expression (Barthel et al., 2005; Kameyama et al., 2011; Rasey et al., 2002). McKinley et al. has tried to reconcile this controversy. They reported that FLT uptake corresponds with tumor proliferation, as a function of thymidine salvage pathway utilization, but not general proliferation as measured by Ki67 expression (McKinley et al., 2013). Furthermore, FLT uptake did not distinguish between tumors which primarily utilized the thymidine salvage pathway (TK1) and those which utilized the de novo thymidine pathway (McKinley et al., 2013).

Despite the numerous studies which have shown FLT-PET's prognostic potential and possible advantages over FDG-PET, we still do not adequately understand the connection between proliferation and FLT-PET. Unless resolved with a clear consensus, this controversy will continue to bar FLT-PET from being utilized clinically.

4 Conclusion

Overall, TK1 is a clinically relevant cancer biomarker which is significantly elevated in serum and tissue of cancer patients. Structurally, TK1 is primarily found as an active tetramer or dimer of dimers. As an active tetramer, TK1 is responsible for converting dT to dTMP in a cell-cycle dependent manner. By extension, TK1 is also responsible for maintaining adequate dTTP levels for DNA synthesis. In tumor cells TK1 plays a pivotal role in DNA repair and affects a tumor's sensitivity to chemotherapy treatment. During the cell cycle, TK1 rapidly increases in late G_1 and peaks in S phase. This rapid increase is primarily due to ATP availability and a concentration-dependent dimer to tetramer switch. Following the S phase peak, TK1 is rapidly degraded by the APC/C-Cdh1 complex which recognizes a KEN box on the c-terminus of TK1.

In malignancy, TK1 exits the cell as a very stable, high molecular weight form which appears to be a TK1 aggregate, 3-7 times larger than active TK1. TK1 is significantly elevated early in the progression of normal cells to malignancy. In fact, in serum and tumors, TK1 is found elevated in some cases of pre-cancer. Furthermore, TK1 is elevated in serum and tumor tissue of virtually all types of cancer. In addition to the diagnostic potential of TK1, TK1 also has beneficial prognostic potential. In particular, high sTK1 or tTK1 levels are associated with worse prognosis, including shorter survival and an increased risk for recurrence. In fact, sTK1 elevates 1-9 months prior to the clinical manifestation of recurrence. As a tool for monitoring a patient's response to treatment, sTK1 decreases significantly in patients with complete response or partial response. On the other hand, sTK1 continues to increase in patients who continue to see disease progression or recurrence. In addition to diagnostic and prognostic potential, TK1 also has limited therapeutic potential. Although most of its therapeutic potential lies with HSV-TK as a suicide gene, TK1 can also be used as a

means of re-sensitizing tumors to chemotherapeutic agents. Finally, TK1 is also used as a potential imaging tool through FLT-PET, as a means of determining the extent of a tumor's proliferation. Clearly, TK1 has vast clinical potential, especially as a screening and monitoring tool for cancer patients. As more accurate and robust methods of detection for TK1 arise, TK1 will no doubt be a powerful clinical tool in the coming years.

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Research Chapter 1: Thymidine Kinase 1 Upregulation is an Early Event in Breast Tumor Formation

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Abstract: Prognostic markers play an important role in our understanding of tumors and how to treat them. Thymidine kinase 1 (TK1), a proliferation marker involved in DNA repair, has been shown to have independent prognostic potential. This prognostic potential includes the novel concept that upregulation of serum TK1 levels is an early event in cancer development. This same effect may also be seen in tumor tissue. In order to demonstrate that TK1 upregulation is an early event in tumor tissue formation, tissue arrays were obtained and stained for TK1 by immunohistochemistry. Using a progressive breast tissue array, precancerous tissue including breast adenosis, simple hyperplasia, and atypical hyperplasia stained positive for TK1 expression. Different stages of breast carcinoma tissue also stained positive for TK1 including nonspecific infiltrating duct, infiltrating lobular, and infiltrating duct with lymph node metastasis carcinomas. This indicates that TK1 upregulation is an early event in breast carcinoma development, and may be useful in identifying precancerous tissue. Further work is needed to better understand the differences seen between TK1 positive and negative tissues.

1. Introduction

Studies have shown that the early detection of breast cancer leads to better patient prognosis and a greater five-year survival rate. Diagnostic and prognostic markers play a key role in classifying tumors and determining the best treatment plan for a patient. The most widely used and established prognostic markers for breast cancer recurrence are tumor size, tumor grade, lymph node involvement, and tumor hormone receptor status. These indicators, although well established, are all related to tumor aggressiveness. Recent evidence has shown that proliferation markers, such as Ki-67 and proliferating cell nuclear antigen (PCNA), may have independent prognostic value [1–3]. Although these proliferation markers have potential, recent studies indicate that thymidine kinase 1 (TK1), another marker associated with proliferation, may be a better prognostic marker than either Ki-67 or PCNA [4, 5].

Thymidine kinase 1 (TK1) has been studied extensively, primarily as a diagnostic biomarker for a variety of cancer types. TK1 is a nucleotide salvage pathway repair enzyme that is primarily responsible for the phosphorylation of thymidine to thymidine monophosphate. TK1 is associated with proliferating cells and is primarily elevated during S phase [6, 7]. As a biomarker, higher serum TK1 activity levels correlate with a more advanced cancer stage and grade [8–10]. Serum TK1 levels also show prognostic potential as their levels help predict future relapse at the time of primary diagnosis in breast and colorectal cancer patients [11, 12].

Similar trends have been found between tumor tissue and TK1 expression levels. One study demonstrated that breast cancer patients who later showed recurrence initially had higher primary tumor TK1 levels when compared to those patients who did not show recurrence [13]. Furthermore, breast cancer patients with either high or intermediate TK1

activity in their tumors showed rapid disease progression and poorer prognosis as compared to patients with low TK1 activity in their tumors [14]. Tumor TK1 levels, similar to serum TK1 levels, also correlate with both stage and grade [15]. Tumor TK1 has also been compared with both Ki-67 and PCNA. Although there is a significant correlation between PCNA and TK1 staining of breast cancer tissue, TK1 showed a significant correlation with stage and grade while PCNA did not, indicating that TK1 might be a more accurate marker for diagnosis and prognosis [4]. Similarly, there is a significant correlation between Ki-67 and TK1 in breast cancer tissue when compared to normal tissue; however, due to early upregulation of TK1 as compared with Ki-67, TK1 may be a more accurate prognostic marker [16, 17].

TK1 upregulation as an early event of cancer is a novel concept that has been addressed by only a few recent studies. One such study involving a health screening of 8,135 people found that 89.2% of persons with elevated serum TK1 levels had diseases linked to risk for pre-/early cancerous progression, including one individual who developed liver carcinoma 13 months after the health screening [18]. Similar studies have also shown that recurrence can be detected by elevated serum TK1 levels as early as 1–6 months before the clinical onset of relapse [19]. These studies show the early nature of serum TK1 levels in tumor development. This study seeks to determine whether, similarly to serum TK1, tumor TK1 upregulation is an early event in tumor development and may aid in the identification of precancerous tissue.

2. Materials and Methods

2.1. Patients and Specimens

Tissue arrays containing tissue from normal ($n = 56$), adenosis ($n = 22$), and breast carcinoma ($n = 97$) patients as well as a progressive breast array (Cybrdi Inc., Frederick MD) were analyzed for TK1 expression. Breast carcinoma tissue included simple carcinoma ($n = 30$), infiltrating duct carcinoma ($n = 41$), medullary carcinoma ($n = 12$), scirrhous carcinoma ($n = 11$), and infiltrating lobular carcinoma ($n = 3$).

2.2. Immunohistochemistry

Tissue arrays were stained using an anti-TK1 mouse monoclonal antibody (CB001), which we previously demonstrated to be highly specific to TK1 [20]. Using this antibody, histological slides were stained using the following procedure. Briefly, formalin-fixed paraffin-embedded specimens were prepared by deparaffinization and rehydration. To retrieve antigenicities of TK1, specimens were boiled in 0.01 M sodium citrate buffer (pH 6.0) for 12 minutes and allowed to cool at room temperature for 20 minutes. The endogenous peroxidase activity was blocked by immersion in 3% H_2O_2 in methyl alcohol at room temperature for 20 minutes. The slides were then washed in phosphate-buffered saline (PBS; pH 7.2) and blocked in 10% normal horse serum for 30 minutes. After

blocking, the slides were incubated at room temperature for 3 hours with either anti-TK1 mouse monoclonal antibody (diluted 1 : 100) or isotype control (0.6 μg/μL, mouse IgG, Upstate Company, 12-371). Slides were washed with PBS and then incubated with a biotin-conjugated anti-mouse secondary antibody (ABC kit, Vector Lab Inc.) at room temperature for 30 minutes. After PBS washing, slides were incubated for 30 minutes, at room temperature, with Streptavidin-Peroxidase (ABC Kit, Vector Lab Inc.) and then washed again in PBS. Diaminobenzidine (Vector Lab Inc.) was used as a chromagen, and the slides were counterstained with haematoxylin.

2.3. Statistical Analysis and Scoring

Specimens were scored by three pathologists, and a consensus score of positive, weak positive, or negative was compiled. A positive score indicated cytoplasmic staining of TK1 in 5–25% of tumor cells. If some signal was detected but was insignificant when compared to the isotype control, it was given a weak positive score. A negative score indicated no staining. All blood vessels and fibrous tissue cores were excluded from statistical analysis. A chi-square test of independence was applied to compare the scores of normal and malignant tissues. Due to the limited number of cases in the progressive breast array, no statistical analysis on this array could be performed. Differences with $P < 0.05$ (two-sided) were regarded as statistically significant.

3. Results and Discussion

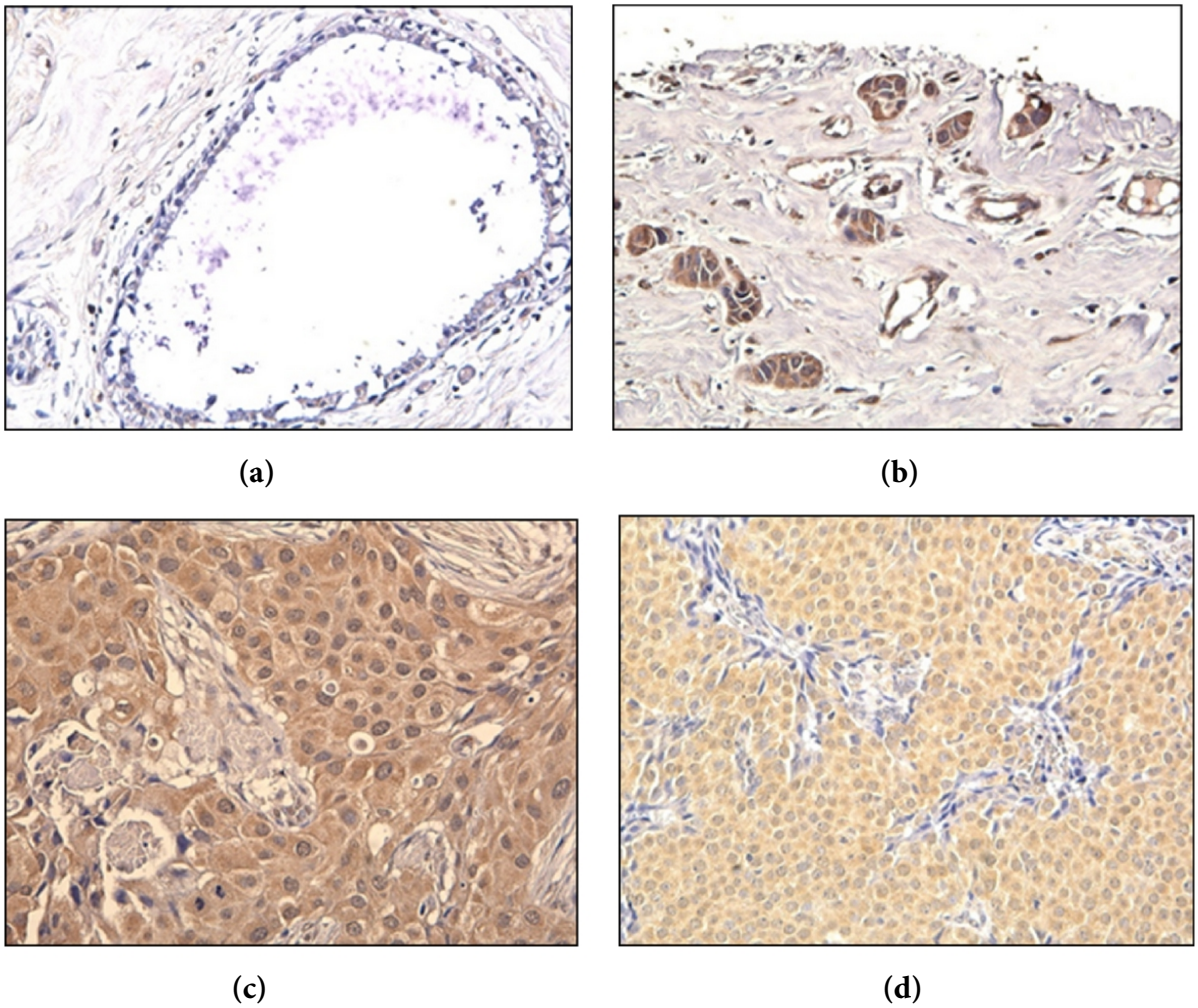
TK1 expression was found to be significantly different ($P < 0.001$) between normal breast and breast carcinoma tissue. A total of 73 breast carcinoma tissues (79%) were positive for TK1 expression while only 18 normal breast tissues (36%) scored positive. Breast tissue was also stained using an isotype control (mouse IgG, Upstate Company), and all breast tissue was found to be negative. Since these normal tissues were retrieved from the margins around a tumor and were considered pathologically normal, we sought to determine if tissue from noncancerous individuals yielded similar results. Interestingly, there was no TK1 staining in any breast tissue obtained from noncancerous individuals, called FDA-approved true normal tissue (data not shown). Therefore, these 18 TK1 positive normal tumor margins may not be false-positive results, but rather precarcinoma tissue, which is considered pathologically normal tissue by current standards. Further work is needed to better understand these potential differences.

TK1 expression was also found to be significantly different ($P = 0.013$) between the different types of breast carcinoma tissue. A chi-square test of independence was applied to compare the scores of the various types of breast carcinoma tissue. The Pearson chi-square value was 22.452, using 10 degrees of freedom, and the two-sided P-value was 0.013. The results are summarized in Table 1 and typical staining can been seen in Figure 1. In summary, infiltrating lobular carcinoma and scirrhous carcinoma tissues all stained positive for TK1, while 66–83% of simple, infiltrating duct, and medullary

carcinoma tissues stained positive for TK1. Further studies with larger sample sizes may further elucidate the differences between these tissue types.

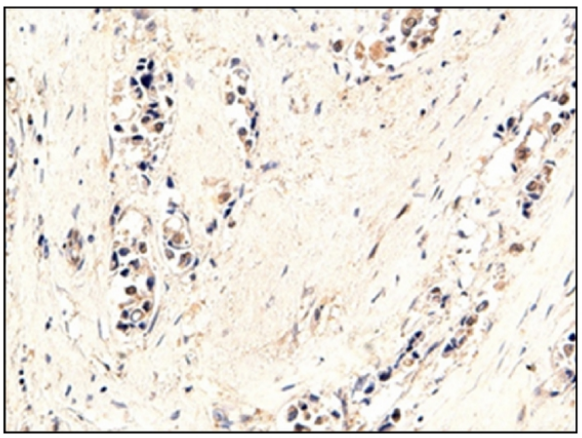
Table 1: TK1 scoring of different types of carcinoma tissue.

Diagnosis	Negative	Weak positive	Positive	Total
Simple carcinoma	3	2	25	30
Infiltrating duct carcinoma	11	4	26	41
Medullary carcinoma	4	0	8	12
Scirrhous carcinoma	0	0	11	11
Infiltrating lobular carcinoma	0	0	3	3
Total	18	6	73	97



(a) **(b)**

(c) **(d)**



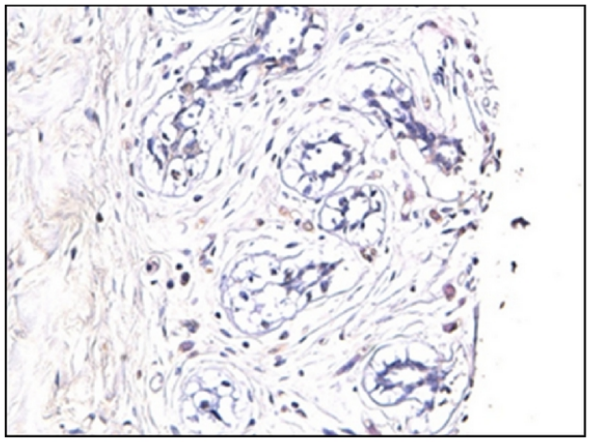
(e)

FIGURE 1: TK1 breast tissue staining. (a) No TK1 staining was found in most normal duct tissue. TK1 positive staining could be found in the cytoplasm of tumor cells of (b) simple carcinoma, (c) infiltrating duct carcinoma, (d) medullary carcinoma, and (e) sclerosing carcinoma tissues.

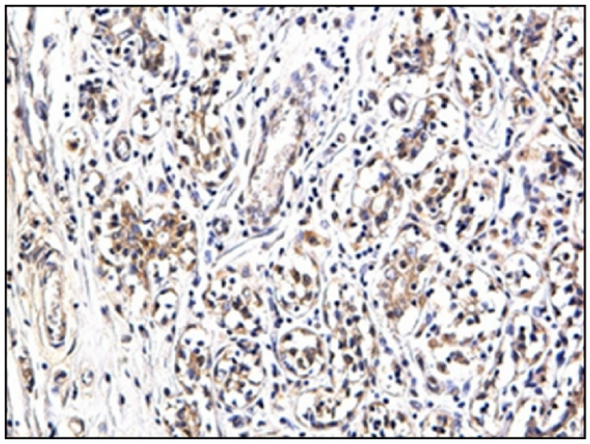
In addition to the TK1 positive breast carcinoma tissues, 4 breast adenosis tissues (22%) were also found to be positive for TK1 expression. These 4 positive precancerous tissues were the first indication that TK1 expression may be an early event in tumor development. To pursue this hypothesis, we obtained a progressive breast array. This progressive breast array included tissue from different tumor developmental stages, such as normal, adenosis, and atypical hyperplasia, moderate atypical hyperplasia, severe hyperplasia, nonspecific infiltrating duct carcinoma, infiltrating lobular carcinoma, and infiltrating duct carcinoma with lymph node metastasis. The results are summarized in Table 2 and typical staining can be seen in Figure 2. The proliferating epithelial cells of some cases of breast adenosis were positive for TK1 expression as well as breast tissue with simple or atypical hyperplasia. As previously seen, most breast carcinoma tissues were also positive for TK1 expression while no breast tissue stained positive with an isotype control. It appears from this progressive array that since TK1 is found in precancerous tissue, TK1 upregulation is an early event in breast tumor development. These results support the previous conclusion that in some cases, there may be a difference between true normal tissue from noncancerous patients and the pathologically normal tumor margins. Further studies are needed to elucidate the differences between both the normal tumor margins and precancerous tissues that were positive for TK1 and those that were negative. Perhaps, the prognostic value of TK1 may be of help in identifying those precancerous tissues which are of greatest risk to the patient. Therefore, TK1 expression is an early event in tumor development and may aid in the identification of precancerous tissue.

Table 2: TK1 scoring of breast progressive array.

Pathological types of breast tissues	Negative/positive
Normal tissues	−
Breast adenosis	−
Sclerosing adenosis	−/+
Atypical hyperplasia	−/+
Infiltrating lobular carcinoma	+
Infiltrating carcinoma	+
Infiltrating carcinoma with lymph node metastasis	+



(a)



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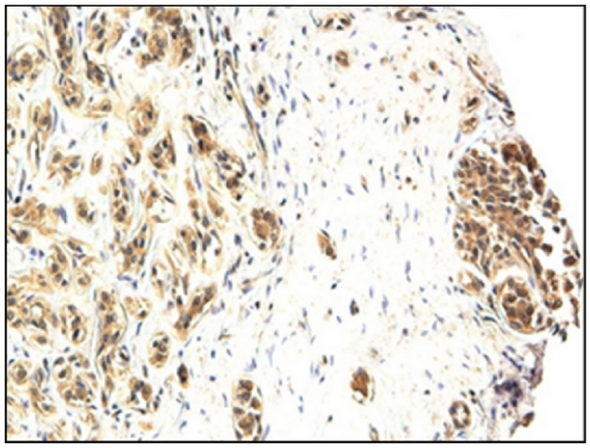

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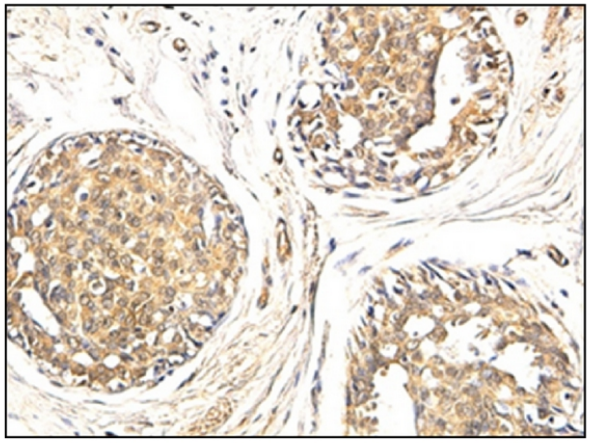

(d)

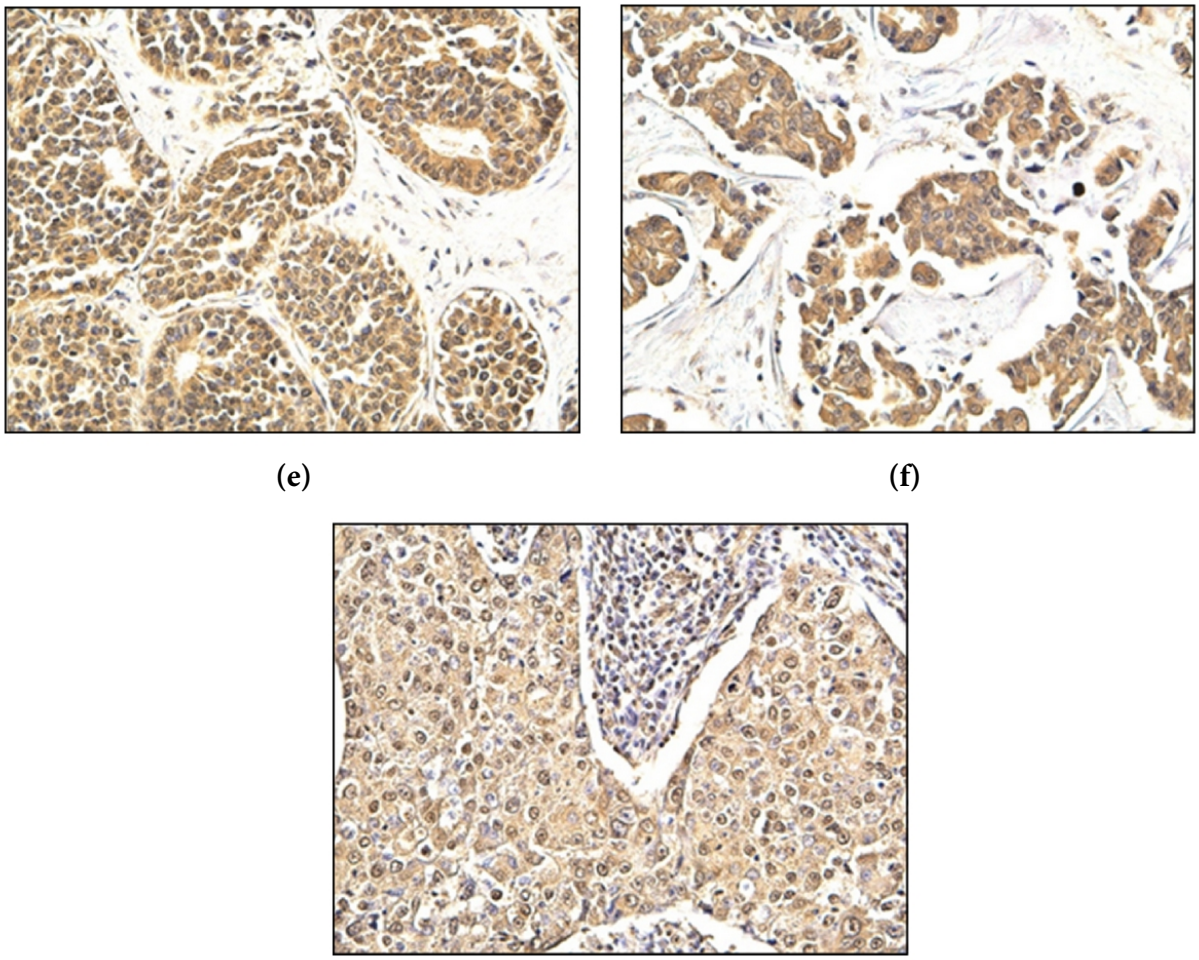

FIGURE 2: Breast progressive array staining. (a) No TK1 staining was found in normal lobule breast tissue. However, TK1 staining was found in proliferating duct epithelial cells of precancerous tissue including, (b) breast adenosis, (c) breast adenosis with mild atypical hyperplasia of duct epithelium, and (d) moderate atypical hyperplasia of duct epithelium. Positive TK1 staining in the cytoplasm of tumor cells was also found in cancerous tissue such as, (e) intraductal carcinoma, (f) infiltrating duct carcinoma, and (g) medullary carcinoma tissues.

4. Conclusion

The aim of this study was to determine if TK1 upregulation is an early event in tumor development. From the progressive breast array, it can be seen that in many cases of breast cancer, TK1 is upregulated in precancerous tissue and remains elevated in correlation to cancer stage. This confirms earlier research that indicated that elevated TK1 levels correlated with early recurrence. Although not elevated in all tumors, TK1 appears to be upregulated as an early event in most tumors and therefore can possibly be used in connection with other diagnostic and prognostic techniques to improve patient

outcome. These results also indicate that the TK1 positive pathologically normal tumor margins may in fact be tumor cells that have escaped pathological identification. This preliminary research may indicate that TK1 can be used to identify possible malignant cells, which have evaded pathological detection during surgical removal. Unfortunately due to the anonymity of these patient samples, we have been unable to determine if TK1 positive tumor margins are of clinical significance. Further research would be required to establish if these TK1 positive cells are in fact a result of the tumor tissue. Overall, it appears that TK1 has diagnostic and prognostic potential in identifying breast tumor tissue as well as precancerous tissues. The ability to identify tumor tissue during the early stages of development is of significant value. Therefore, the histological identification of tumors utilizing TK1 suggests promising prognostic and diagnostic potential in breast cancer tissue.

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Research Chapter 2: Thymidine Kinase 1: A Universal Marker for Cancer

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Abstract: Thymidine Kinase 1 (TK1) is traditionally a serum biomarker which is elevated in the early stages of malignancies. TK1 is a DNA repair enzyme which is typically associated with proliferation. In serum, TK1 is elevated in a stage-like manner and increases as the disease advances. As a well-characterized serum biomarker, relatively little work has been done to establish it as a tumor biomarker. However, tumor TK1 reflects many of the same trends that can be seen with serum TK1. Specifically, tumor TK1 is an early event in tumor progression and increases with grade and stage. This study seeks to demonstrate that, similar to serum TK1, tumor TK1 is a valuable indicator of malignancy in the most common types of cancer in men. In this study we used a highly specific monoclonal antibody to TK1 to histologically stain 10 different types of carcinoma tissue and corresponding normal tissue. We found that TK1 is a good marker for malignancy and is significantly overexpressed in cancers compared to normal controls in lung, colon, prostate, esophagus, stomach, liver, and kidney tissues. Slight differences were found in the staining pattern among various types of lung cancer although virtually all types showed significantly higher TK1 staining compared to normal tissue. Additionally, TK1 expression in prostate carcinoma was significantly higher than from normal tissue, and correlated with an increase in stage. Overall, it is clear that TK1 is a valuable cancer marker in a wide variety of solid tumors and may be considered a universal cancer marker.

1. Introduction

Cancer is the second leading cause of death in the United States but is the number one cause of death in individuals ages 40 to 79 years (Siegel, Naishadham, & Jemal, 2013). In men, the two most prevalent cancer sites which contribute to cancer mortality are lung and prostate. Together these account for approximately 38% of cancer deaths in men (Siegel et al., 2013). Although many biomarkers have been identified in the hopes of reducing cancer mortality rates, few have been successful. However, thymidine kinase 1 (TK1) may have independent potential to reduce cancer mortality rates through early detection.

TK1 is a biomarker which is involved in DNA repair and is upregulated during S phase (He, Skog, & Tribukait, 1991). It is considered a proliferation marker and is often compared with Ki67 and/or proliferating cell nuclear antigen (PCNA). Several studies have shown that TK1 is more useful as a proliferation marker than either Ki67 or PCNA (Guan et al., 2009; He et al., 2004; Z. H. Huang et al., 2012). TK1 has primarily been studied as a diagnostic and prognostic tool found in the serum of cancer patients. TK1 is elevated in a stage-specific manner in a variety of solid and hematological malignancies (Aufderklamm et al., 2012). For example, TK1 has been established as a serum biomarker in breast cancer, lung cancer, colon cancer, malignant melanoma, gastric cancer, non-Hodgkin's lymphoma, chronic lymphocytic leukemia, acute lymphoblastic leukemia, and acute myeloid leukemia in addition to several other cancer types (Li et al., 2010; Liu et al., 2011; O'Neill, Zhang, Li, Fuja, & Murray, 2007; Wu et al., 2013; Xu et al., 2009). TK1 is elevated in the very early stages of cancer and has even been found elevated in the precancerous stages (S. Huang et al., 2011). In fact, in a health screen of 35,365 individuals 0.8% had elevated serum TK1, and 8.8% of those with elevated TK1 went on to develop new malignancies or had progression of their pre-malignancies (Chen et al., 2011). Recent studies have also found that TK1shows similar trends in tumors. In breast cancer patients, TK1 was overexpressed in atypical ductal hyperplasia, precancerous lesions, and also in carcinoma tissue (Alegre, Robison, & O'Neill, 2012; Guan et al., 2009). Although TK1 has been well-characterized as a serum biomarker and similar trends are seen with tumor TK1, this study seeks to compare TK1 expression in a variety of solid tumors. In particular, we compare TK1 expression within the two most common types of cancer in men: prostate and lung, to possibly establish TK1 as a candidate marker for the major forms of cancer in men.

2. Methods

2.1 Patient Samples

All the following arrays were analyzed for TK1 expression using a highly-specific anti-TK1 monoclonal antibody (CB001) (O'Neill, Buckwalter, & Murray, 2001). A multi-organ tissue array containing carcinoma tissue from 9

different organs of 30 individuals and normal controls of 7 different organs from 48 individuals were analyzed for TK1 expression. Carcinoma tissue included liver (n=6), stomach (n=6), esophagus (n=6), kidney (n=6), uterus (n=5), colon (n=6), brain (n=6), muscle (n=6), and lung (n=6). Normal tissue included liver (n=6), stomach (n=6), esophagus (n=6), kidney (n=6), colon (n=6), rectum (n=6), and lung (n=6). Prostate tissue arrays included tissue from prostate carcinoma (n=32), normal prostate (n=17) and prostate hyperplasia (n=6). True normal tissue arrays included tissue from 100 individuals and 34 organs (Cybrdi Inc, Frederick MD). Lung tissue arrays included lung cancer tissue (n=376) from 172 individuals and normal lung tissue (n=63) from 29 individuals (Cybrdi Inc, Frederick MD). Lung cancer tissue included small cell carcinoma (n=37), large cell carcinoma (n=19), adenocarcinoma (n=26), squamous cell carcinoma (n=36), sarcoma (n=2), adenosquamous cell carcinoma (n=4), bronchioloalveolar carcinoma (n=5), mucinous adenocarcinoma (n=1), mesothelioma (n=2), undifferentiated carcinoma (n=4), neuroendocrine carcinoma (n=3), and papillary carcinoma (n=1).

2.2 Immunohistochemistry

Each of the tissue arrays were stained using an anti-TK1 monoclonal antibody (CB001) and an isotype control (mouse IgG, Upstate Company, 12-371). The staining procedure is as previously described (Alegre et al., 2012). Briefly, the tissues were deparafinized and rehydrated. After antigen retrival with 0.01M Sodium Citrate buffer (pH 6.0) and blocking for endogenous peroxidase activity, slides were blocked (10% normal horse serum) and then incubated for 3 hours with CB001 or isotype control (0.6ug/ml). After washing, slides were incubated with biotin-conjugated anti-mouse secondary antibody (Vector Labs Inc.), washed again, and incubated with Streptavidin-Peroxidase (ABC Kit, Vector Labs Inc.). Slides were developed using diaminobenzidine (Vector Labs Inc.) and counterstained with haematoxylin.

2.3 Statistical Analysis and Scoring

Each tissue specimen was scored by three independent pathologists and given a consensus score of negative, weak positive, or positive. Additionally, the lung tissue arrays were scored using a fourth category of strong positive. In each case, a positive score was associated with staining in 5-25% of tumor cells. A weak positive score indicated that although some staining was present, it was insignificant compared to the isotype control. A negative score was associated with no staining. Additionally, a strong positive score for the lung arrays indicated >25% of tumor cells showed TK1 expression. Statistical analysis of the tissue specimens included a chi square test of independence and $p<0.05$ (two-sided) was regarded as statistically significant.

3. Results

3.1 A Universal cancer marker: TK1expression

TK1 expression was evaluated using a highly specific monoclonal antibody in histological slides of 9 different carcinoma tissues from 30 individuals including brain, esophagus, stomach, colon, liver, lung, kidney, uterine, and muscle. TK1 expression was also evaluated in 8 different normal tissues from 48 individuals including esophagus, stomach, liver, uterine, colon, rectum, lung, and kidney. Three independent pathologists scored each tissue and TK1 cytoplasmic staining was detected in hepatocellular carcinoma, clear cell carcinoma of kidney, uterus rhabdomyosarcoma, esophageal squamous cell carcinoma, and adenocarcinoma of the uterus, stomach, colon, and lung (Figure 1). On the other hand, weak positive TK1 expression was found in 3 cases of meningioma indicating that although some signal was present, it was insignificant compared to the isotype control. No TK1 staining was detected in normal esophagus, stomach, and kidney tissue (Figure 1). Weak positive TK1 expression was found in normal lung, liver, colon, and rectum tissues (Figure 1). There was no significant difference between carcinoma and normal uterus tissue. Unfortunately, only two normal uterine tissues were available for this analysis and more tissue is needed to confirm these findings. TK1 expression was significantly higher in tumor tissue of the esophagus, stomach, colon, liver, lung and kidney compared to normal tissue (Table 1). Each of the tissues was also stained with an isotype control (mouse IgG, Upstate Company), and all tumor and normal tissue was negative.

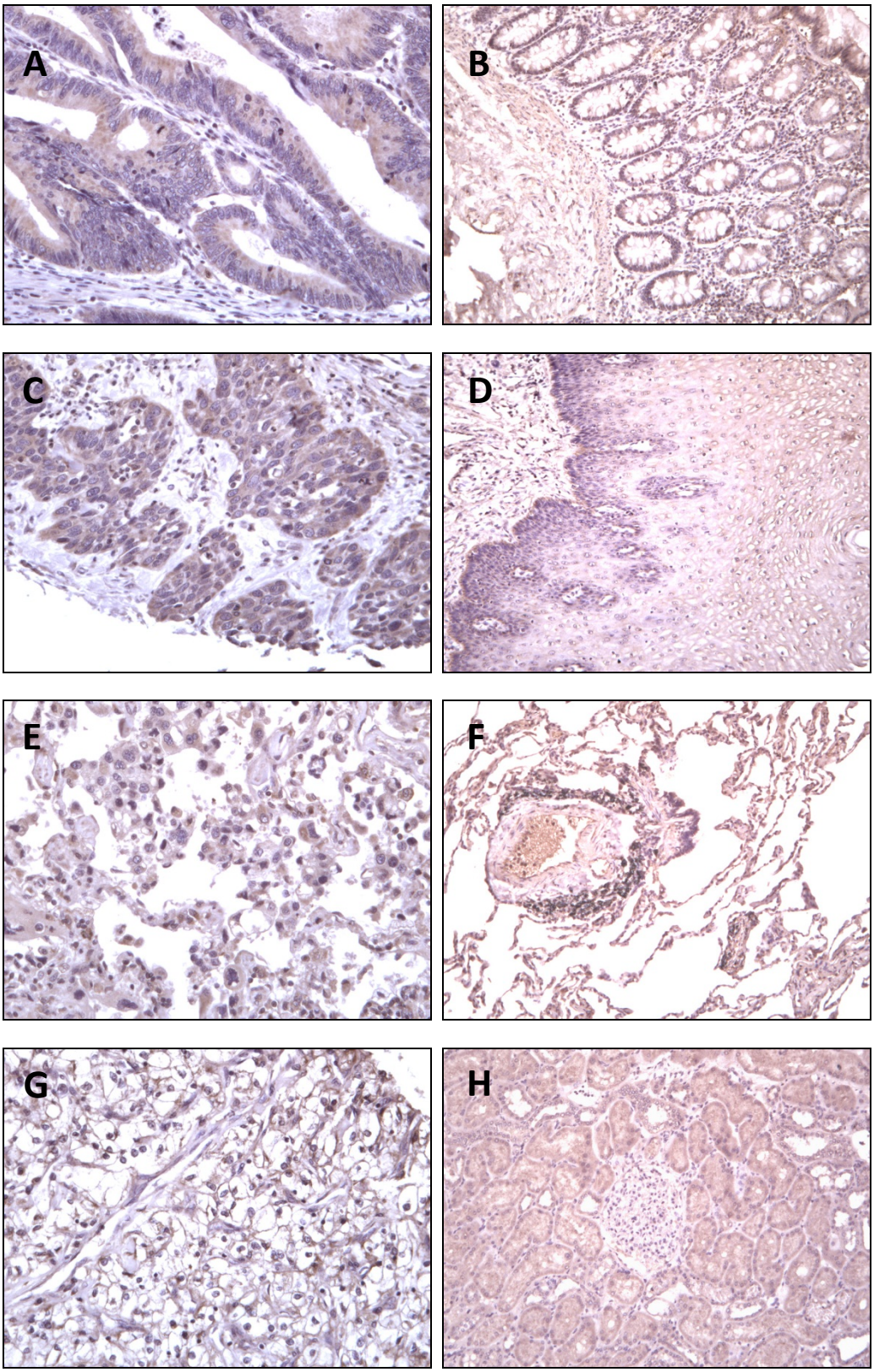

Figure 1: TK1 multi-organ tissue staining.

Cytoplasmic TK1 staining was found in A) colon adenocarcinoma, C) esophageal squamous cell carcinoma, E) lung adenocarcinoma, and G) kidney clear cell carcinoma tissues. None or weak TK1 staining can be found in normal B) colon, D) esophagus, F) lung, and H) kidney tissues.

Table 1: TK1 staining in normal and carcinoma tissue.

		Negative	Weak Positive	Positive	Total	Significance
Liver	Normal	0	6	0	6	0.001
	Carcinoma	0	0	6	6	
Stomach	Normal	6	0	0	6	0.014
	Carcinoma	1	3	2	6	
Esophagus	Normal	4	1	0	5	0.016
	Carcinoma	0	2	4	6	
Kidney	Normal	6	0	0	6	0.001
	Carcinoma	0	0	6	6	
Uterus	Normal	0	0	2	2	0.29
	Carcinoma	0	2	3	5	
Colon	Normal	0	6	0	6	0.014
	Carcinoma	3	1	2	6	
Lung	Normal	0	5	0	5	0.022
	Carcinoma	0	2	4	6	

Interestingly, the normal tissue included in this study was initially taken from the margins around the tumor. These margins from cancer patients were considered normal using current pathological methods. However, recent studies have indicated that the normal microenvironment surrounding a tumor in many cases is altered and has lost its normal regulation (Alegre et al., 2012; Farmaki et al., 2012; Niu et al., 2012). Previous studies have suggested that this may help explain some TK1 positive staining in these normal tumor margins (Alegre et al., 2012). Therefore, we obtained normal tissue from 100 noncancerous individuals, called true normal tissue, which included 34 true normal organs (Cybrdi Inc, Frederick MD). TK1 cytoplasmic staining was found in adrenal gland cells, germ cells of the seminiferous tubules of the testis, and hematopoietic cells of the bone marrow (Figure 2). Weak background staining was seen in tubular cells of the kidney, cardiac and striated muscle cells, salivary gland duct cells, mucous secreting cells in small intestine and stomach, and transitional cells of the ureter and bladder (Figure 2). No other true normal organs stained positive for TK1, including those normal tumor margins which previously showed weak positive staining for TK1.

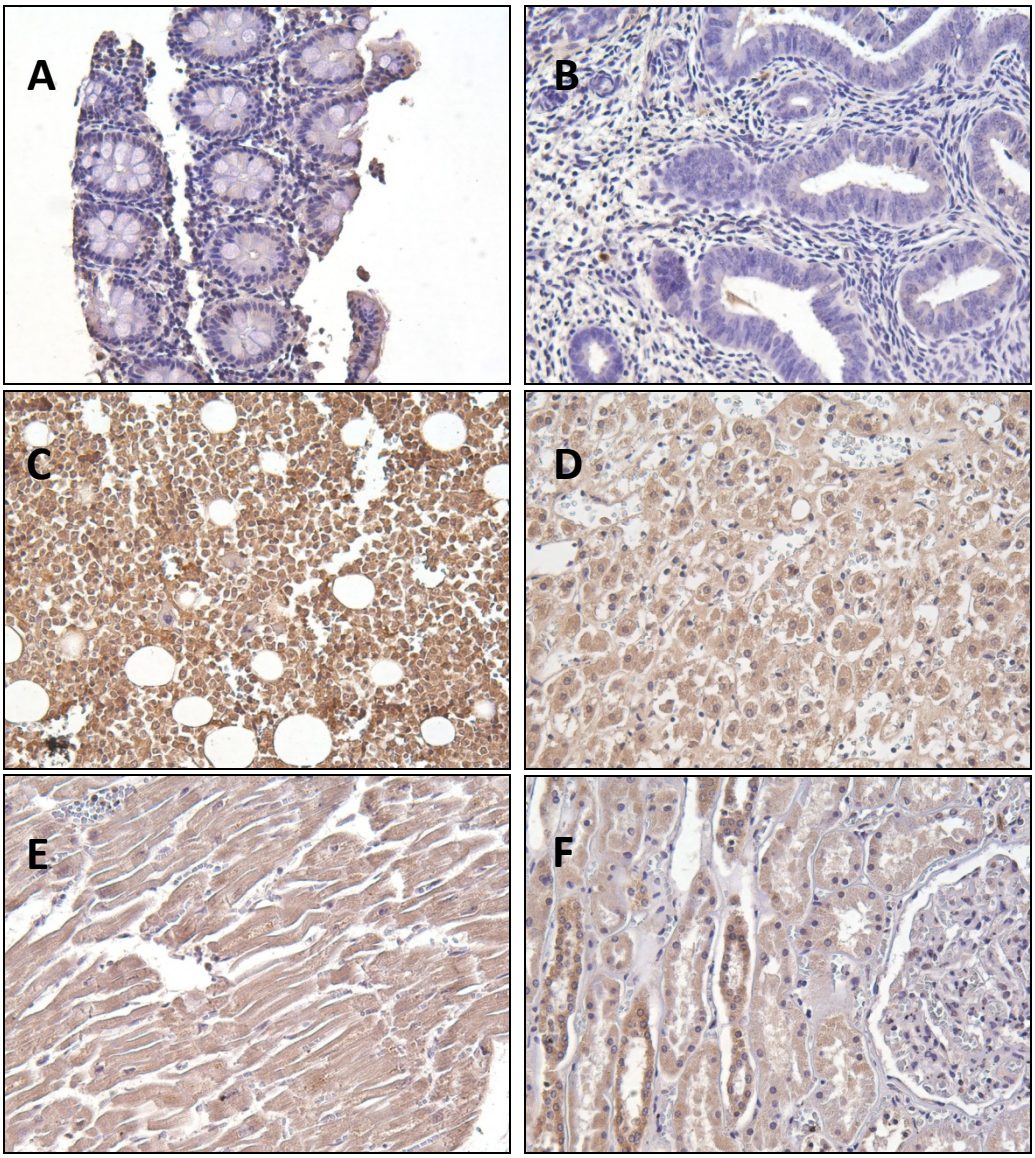

Figure 2: TK1noncancerous true normal staining.
No TK1 staining could be found in A) true normal colon, and B) true normal uterus tissue. Cytoplasmic TK1 staining was found in C) true normal bone marrow and D) true normal adrenal gland. Weak positive TK1 staining was found in E) true normal cardiac tissue and F) true normal kidney tissue.

3.2 A marker for the #1 cancer killer in men: TK1 and lung cancer

Since the majority of cancer mortality in men is due to lung cancer, we investigated the link between TK1 staining and lung cancer more thoroughly. TK1 expression was evaluated in 376 lung cancer tissue cores representing 172 individuals and 63 normal lung cores from 29 individuals. Samples represented a variety of lung cancer diagnoses including small cell carcinoma, large cell carcinoma, adenocarcinoma, squamous cell carcinoma, adenosquamous cell carcinoma, bronchioloalveolar carcinoma, mucinous adenocarcinoma, undifferentiated carcinoma, neuroendocrine carcinoma, papillary carcinoma, mesothelioma, and sarcoma. Combining all lung cancer tissues, there is a highly statistically significant difference between TK1 expression in lung cancer and normal lung tissue ($p<0.0001$). In this study, we only had access to one mucinous adenocarcinoma and one papillary carcinoma, and both cases showed positive TK1 expression, but further samples should be tested to confirm this finding. Neuroendocrine carcinoma tissues were not significantly different in TK1 expression compared to normal lung

tissue. All other lung cancer groups showed significantly higher TK1 expression compared to normal controls including small cell carcinoma, large cell carcinoma, adenocarcinoma, squamous cell carcinoma, undifferentiated carcinoma, mesothelioma, bronchioloalveolar carcinoma, adenosquamous cell carcinoma, and sarcoma (Table 2). There was also a statistically significant difference in TK1 expression between small cell lung cancer and non-small cell lung cancer ($p<0.0001$) in which non-small cell lung cancer exhibited higher TK1 expression. Additionally, squamous cell carcinoma had significantly higher TK1 expression compared to adenocarcinoma tissues ($p<0.0001$), but squamous cell carcinoma was not significantly different from large cell carcinoma. Although each of the individual lung cancer diagnosis groups differed slightly, all were significantly higher than normal lung tissue, indicating that TK1 may be a valuable marker for lung cancer.

Table 2: TK1 staining in various types of lung cancer.

	Negative	Weak Positive	Positive	Strong Positive	Total	Significance
Small Cell Carcinoma	11	26	0	0	37	0.0018
Large Cell Carcinoma	5	6	1	7	19	<0.0001
Adenocarcinoma	13	5	8	0	26	<0.0001
Squamous Cell Carcinoma	3	10	18	5	36	<0.0001
Bronchioloalveolar Carcinoma	0	3	2	0	5	<0.0001
Adenosquamous Cell Carcinoma	0	1	2	1	4	<0.0001
Undifferentiated Carcinoma	0	2	2	0	4	0.0002
Neuroendocrine Carcinoma	0	0	2	1	3	0.0755
Sarcoma	0	1	1	0	2	0.025
Mesothelioma	0	0	2	0	2	0.0016
Mucinous Adenocarcinoma	0	0	1	0	1	-
Papillary Carcinoma	0	0	1	0	1	-
Normal Lung	24	4	1	0	29	-

3.3 A marker for the #2 cancer killer in men: TK1 and prostate cancer

Prostate cancer is responsible for a large portion of cancer mortality in men, second only to lung cancer. Due to this high prevalence in both mortality and incidence, we sought to determine whether TK1 histological staining could also be used as a marker for prostate cancer. TK1 expression was evaluated in 57 prostate tissues which represented 20 individuals. Each individual was represented by 3 different tissue cores including normal, hyperplasia, or carcinoma tissues. Each tissue was also stained with an isotype control (mouse IgG, Upstate Company) and all these isotype-stained tissues were negative. The majority of prostate adenocarcinoma tissue exhibited TK1 cytoplasmic positive staining which was significantly different compared to either normal or hyperplasia tissues ($p<0.0001$) (Table 3). Additionally, TK1 expression increased as prostate adenocarcinoma advanced from grade 1 to grade 3 ($p=0.004$) (Figure 3). This confirms our previous findings that TK1 is a valuable marker of malignancy and is expressed in the very early stages of cancer.

Table 3: TK1 staining in prostate cancer.

	Negative	Positive	Total	Significance
Normal	9	8	17	
Hyperplasia	6	0	6	
Adenocarcinoma	5	27	32	<0.0001
Adenocarcinoma grade 1	0	1	1	
Adenocarcinoma grade 2	3	4	7	
Adenocarcinoma grade 3	0	22	22	0.004

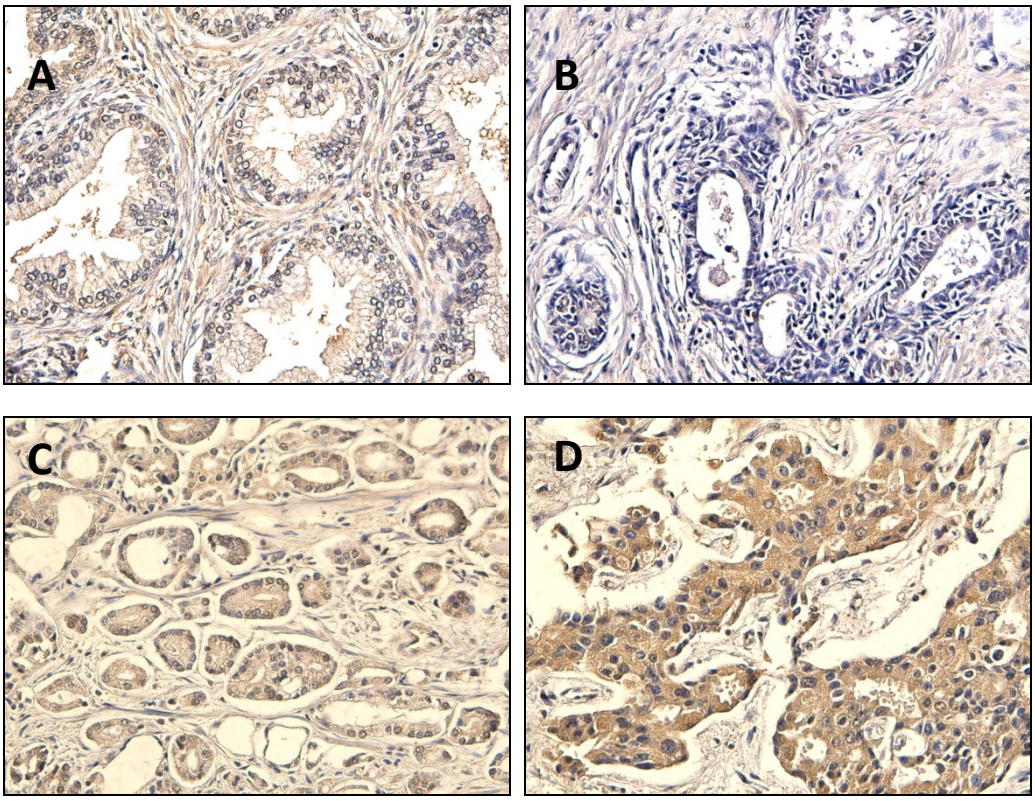

Figure 3: TK1 staining in prostate cancer.
No TK1 staining was found in A) normal prostate and B) prostate hyperplasia. Cytoplasmic TK1 staining was found in C) prostate carcinoma grade 2 and D) prostate carcinoma grade 3. There was a significant increase in TK1 expression from prostate carcinoma grade 1 to grade 3.

4. Discussion

This is the first study to do an overall comparison and analysis of TK1 expression among a variety of malignant tissues. It is clear that TK1 is a good marker for malignancy as it is significantly increased in a variety of carcinoma tissues. For example, as the data in Table 1 indicates, there is a highly significant difference between normal and carcinoma tissues for virtually all organ types. From this we determined that TK1 is a good marker of malignancy in at least seven of the most common types of cancer, including lung, colon, prostate, esophagus, stomach, liver, and kidney. Typically, if TK1 is expressed in normal tissue, it is very weak and insignificant compared to the isotype control. Although there was no significant difference in TK1 expression between normal and carcinoma uterine tissues in our study, several other studies have demonstrated that TK1 can be used as a marker for uterine malignancy (Shintani, Urano, Takakuwa, Kuroda, & Kamoshidai, 2010). Additionally, TK1 as a marker for breast cancer and hematological malignancies, has been well-established by previous studies (Aufderklamm et al., 2012). Together, this indicates that TK1 appears to be a good universal marker for cancer among both solid and hematological malignancies.

Previous studies have demonstrated that TK1 overexpression is an early event in cancer, both in the serum of cancer patients and in primary tumors (Guan et al., 2009; S. Huang et al., 2011). Interestingly, our study revealed that TK1 is expressed in some normal tissue (Table 1). However, this tissue was taken from the tumor margins and considered normal using current pathology standards. On the other hand, the same organ tissue taken from noncancerous individuals, called true normal tissue, did not express TK1. Due to the early diagnostic nature of TK1, it may be that these normal tumor margins may in fact be precancerous lesions which are considered normal by current pathology standards. Perhaps, these cells which normally remain undetected may play a role in the development of recurrent lesions. It would be interesting to follow the patients with TK1 positive tumor margins to determine if those patients

later relapsed or showed worse prognosis. Unfortunately, no follow-up data was available and we could not determine the clinical significance of TK1 positive tumor margins.

Of particular note, TK1 expression is a good marker for both lung and prostate cancer, the two most frequently diagnosed and leading causes of cancer-related deaths in men (Siegel et al., 2013). Although there are slight differences in the staining patterns of lung cancer, virtually all types of lung cancer had higher TK1 expression which was highly significantly different compared to normal lung tissues (Table 2). Prostate cancer tissues showed similar trends and even showed increasing TK1 expression from grade 1 to grade 3 prostate carcinoma tissues.

5. Conclusion

Our results indicate that TK1 expression is significantly higher in malignancy compared to normal tissue. TK1 appears to have potential as a tumor marker in a wide variety of malignancies, including at least seven of the most common types, and thus may be a useful universal cancer marker.

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Research Chapter 3: Serum and Sputum detection of Thymidine Kinase 1 as a means of early detection of lung cancer

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Abstract: Introduction—Lung cancer is known for its late-stage diagnosis, ultimately leading to high mortality. Thymidine kinase 1 (TK1) is an early detection biomarker, elevated in malignancies including lung cancer. Elevated TK1 is an early event which is associated with risk of malignant progression. In this study, we propose a sensitive and specific TK1 immunoassay for the early detection of lung cancer.

Methods—Forty pre-operable lung cancer and 18 normal serums were collected. TK1 levels were determined by the traditional TK radioassay and a novel TK1 enzyme-linked immunosorbent assay (ELISA). Additionally, 17 sputum samples were analyzed for TK1 activity.

Results—TK1 was significantly elevated in all lung cancer and stage 1, node-negative patients compared to controls ($p<0.001$ and $p=0.009$, respectively). The best cut-off value was 4.9 nM. This gave an area under the curve of 0.792 and 0.828 for early stage and all lung cancer respectively. The sensitivity and specificity for all lung cancer patients was 82.4 and 83.3, respectively. There was no significant difference in TK1 activity between lung cancer and healthy controls. This indicated that TK1 concentration is a more sensitive and accurate indicator of lung cancer than TK1 activity. TK1 activity is significantly higher in lung cancer sputum compared to controls, demonstrating a possible TK1 lung cancer specific test.

Conclusions—Overall, TK1 is significantly elevated in the lung cancer serum and sputum of patients. This novel TK1 ELISA is both sensitive and specific for early stage and advanced lung cancer. This assay may aid in the early detection of lung cancer.

Introduction

Lung cancer accounts for the most cancer-related deaths in men and women [1]. Currently there are no FDA-approved biomarkers for the early detection or monitoring of lung cancer [2]. The only recommended early detection tool for lung cancer is a low-dose spiral CT scan. The National Lung Screening Trial reported 20% reduced mortality for high-risk individuals screened with spiral CT scans [3]. Despite its promising potential, there are many disadvantages to this screening method including cost, invasiveness, and exclusivity. In this study we propose a novel early detection test which may overcome many of these limitations. We demonstrate that this early detection enzyme-linked immune sorbent assay (ELISA) for thymidine kinase 1 (TK1) is highly sensitive and specific for the diagnosis of stage 1, node-negative lung cancer as well as more advanced lung cancer patients.

TK1 is a well-established cancer biomarker which is elevated in the serum and tumor tissue of many hematological and solid tumors including lung cancer [4-6]. TK1 is efficacious as both a diagnostic and prognostic tool since changes in serum TK1 levels reflect a patient's response to treatment, and risk for recurrence [7-9]. TK1 is elevated in the very early stages of malignancy. In fact, in a health screen of 8,135 individuals, 1.1% of individuals had elevated TK1 levels in which nearly 90% of those with elevated TK1 had pre-cancerous diseases [10]. Included in those non-cancerous individuals with elevated TK1 was one individual who developed liver carcinoma 13 months following the reported high TK1 level. Several other studies have confirmed that TK1 is elevated in pre-malignancy. Guan et al. demonstrated that TK1 is significantly elevated in atypical ductal hyperplasia (ADH), ductal carcinoma in situ, and invasive ductal carcinoma compared to normal breast tissue [11, 12]. However, they reported that TK1 was not significantly elevated in usual ductal hyperplasia tissue (UDH). Since TK1 was elevated in some pre-cancerous diseases such as ADH but not others, UDH, this indicated that perhaps elevated

TK1 is associated with pre-cancerous diseases which have an increased risk of progression. This appears to be the case and has been confirmed in several large health screens including one with 35,365 individuals and one with 11,880 individuals [13, 14]. In these health screens, individuals with pre-malignant diseases were found in both the elevated TK1 and normal TK1 groups although a significantly higher percentage of individuals with pre-malignant diseases had elevated TK1. The pre-malignant patients with elevated TK1 were more likely to show disease progression within 5 to 72 months compared to pre-malignant patients with normal TK1 (8.8% vs 0.2%) [14]. Therefore, non-cancerous individuals with elevated TK1 levels had 3-5 fold increased risk of developing malignancies [14].

Lung cancer is frequently diagnosed in the advanced stages, and TK1 is elevated in lung cancer and other malignancies very early. Therefore, we sought to develop a simple TK1 ELISA to help identify lung cancer patients much earlier. We hope this will be an efficacious alternative to the spiral CT scan and help to decrease lung cancer mortality rates. In this study, we demonstrate a sensitive and specific TK1 ELISA in which stage 1, node-negative lung cancer patients and more advanced lung cancer patients have significantly elevated serum TK1 levels compared to healthy individuals. Furthermore, we demonstrate that this TK1 ELISA is more sensitive than traditional methods of detecting TK1 in lung cancer patients.

Materials and Methods

Patient samples. Serum samples from 40 pre-operable lung cancer patients were obtained from University of Colorado medical center. Serum samples from 18 healthy individuals were obtained from community volunteers near Brigham Young University. Additionally, 17 sputum samples (lung cancer sputum, n=15, normal sputum, n=2) were obtained from the University of Colorado. All samples were collected according to IRB guidelines and all patients signed consent forms. Upon collection, samples were immediately stored as aliquots at -80ºC until analysis.

TK1 ELISA. Serum samples were analyzed using a previously described direct ELISA protocol with slight modifications [15]. The anti-TK1 antibody used was a highly specific mouse monoclonal antibody (produced by our lab) which reacts against an internal fragment of TK1, amino acids 46-145, designated A74. Briefly, 50 µl samples were diluted in carbonate buffer, pH 9.6 and allowed to absorb to 96-well ELISA plates overnight at 4ºC. Wells were then washed four times with phosphate-buffered saline (PBS), pH 7.4 and blocked with 2% BSA diluted in T20 PBS Blocking Buffer (Thermo Fisher Scientific) for one hour at room temperature. Wells were again washed and then incubated for one hour at room temperature with HRP conjugated anti-TK1 antibody diluted in block. Wells were again washed and developed with 1-Step Ultra TMB (Thermo Fisher Scientific) and quenched with sulfuric acid. Samples were analyzed using Synergy HT Microplate Reader (Bio-Tek) at 450 nm. Samples were run in duplicate and confirmed with at least 2 independent experiments. The standard curve was constructed using recombinant human TK1 at varying concentrations diluted in carbonate buffer. The average of the serum samples and the recombinant TK1 standard curve was used to calculate TK1 concentration in the serum samples.

TK1 Radioassay. The TK radioassay was optimized for human serum samples and run as previously described [16]. The reaction mix contained 10 mM Tris-HCl pH 7.6, 2 mM DTT, 5 mM $MgCl_2$, 5 mM NaF, 5 mM ATP, and 5 µM [^{3}H]-dThd. Samples were run as quadruplets and the data was confirmed with at least 2 independent experiments. The average of all runs was used for statistical analysis. Sputum samples were also analyzed using the TK radioassay. Since cell extracts often have both TK1 and TK2 activity, sputum samples were run separately with ATP and CTP. ATP is utilized by both TK1 and TK2 while CTP is only utilized by TK2 [17]. Therefore, TK1 activity in sputum samples is reported as % TK1. Since TK2 is not found in significant quantities in serum samples, this is not necessary for TK1 activity in serum [16].

TK1 Dot Blot. Serial dilutions of serum or recombinant TK1 (10 µl) were applied to a nitrocellulose membrane (Bio-Rad) and allowed to dry for 1 hour at room temperature. The membrane was then blocked with 5% non-fat dry milk (NFDM) diluted in tris-buffered saline (TBS) for one hour at room temperature. Following 3 washes with 0.1% Tween T20 in TBS (TBS-T), the membrane was incubated with A74, a highly specific anti-TK1 monoclonal antibody, diluted in block for one hour at room temperature. The membrane was washed five times with TBS-T and incubated with goat anti-mouse HRP conjugated secondary antibody absorbed against human, bovine, horse, rabbit, and swine (Jackson ImmunoResearch) for one hour at room temperature. The membrane was washed again and developed using Immun-Star WesternC Chemiluminescence kit (Bio-Rad).

Immunohistochemistry. TK1 staining of lung cancer and normal tissue was performed as previously described [12]. Briefly, tissue slides were deparaffinized, rehydraded and antigenicities were retrieved using 0.01M sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked, and washed prior to incubation with 10% normal horse serum. Primary antibody, a highly specific anti-TK1 monoclonal antibody (CB001), was added for 3 hours at room temperature. Following the primary antibody, slides were washed, incubated with biotin-conjugated secondary antibody, washed and then incubated with streptavidin-peroxidase (ABC kit, Vector Labs Inc). Slides were then washed, developed using diaminobenzidine (Vector Labs Inc), and counterstained with haematoxylin.

Statistical Analysis. The diagnostic power of this TK1 ELISA was determined by analyzing the area under the receiver operating characteristics (ROC) curve. The best cut-off value was determined by maximizing sensitivity and specificity. The data is presented as mean ± the standard deviation (SD) and median ± interquartile range (IQR). Student t-test and Wilcoxon rank-sum test were used to determine statistical significance, which was set at $p< 0.05$. Sensitivity, specificity, positive predictive value, negative predictive value, and odds ratio (± 95% CI) were reported. All analyses were performed using SPSS software.

Results

Patient characteristics

Forty pre-operable lung cancer patients' sera were collected prior to any treatment. Sixteen patients were stage 1, node-negative (pT1a and pT1b), and 18 patients were diagnosed with stage 2 lung cancer (Table 1). Additionally, 6 patients had secondary lung tumors arising from the colon, esophagus, cervix, or prostate. Most lung tumors were adenocarcinomas (n=16) although several patients were diagnosed with squamous cell (n=8) and adenosquamous cell carcinoma (n=2) (Table 1). The median age of lung cancer patients was 68, range 22-88. There was a fairly high percentage of never smokers (n=9) in our data-set compared to the national average of never-smoker lung cancer patients, 28% and 10-15% respectively [18]. We chose to focus on pre-operable, typically stage 1 and 2, lung cancer patients as a means of detecting lung cancer in its early stages. Overall, we feel our patient population represents a good sampling of early stage, pre-operable lung cancer patients.

Table 1: Lung cancer patient characteristics

Diagnosis:	Adenocarcinoma	16
	Squamous cell carcinoma	8
	Adenosquamous cell carcinoma	2
	Other lung cancer	8
	Metastasis to lung	6
	Non-cancer	18

Lung cancer by stage:	Stage 1	16
	Stage 2	18
	Metastasis to lung	6
Age and Sex:	Median age in years (range)	68 (22-88)
	Female	17
	Male	23
Cancer patient	Never smoker	9
smoking history:	Former smoker	14
	Current smoker	17

A74: anti-TK1 antibody specificity

In order to confirm that A74 can accurately detect native TK1 in tumor tissue as well as serum, we stained lung adenocarcinoma and squamous cell carcinoma tissues for TK1 expression using a highly specific monoclonal antibody (CB001) appropriate for TK1 immunohistochemical detection (Figure 1). TK1 expression was clearly elevated in lung cancer tissue with minimal or no staining in normal lung tissue. This confirmed several other studies which have reported significantly elevated TK1 expression in lung carcinoma tissue, including pT1 adenocarcinoma tissue, compared to normal lung [4, 6]. Furthermore, we determined through dot blot that A74 TK1 antibody detected native recombinant TK1 as well as native cancer serum TK1 (Figure 1). Native western blot analysis confirmed that A74 detected native serum TK1 in its expected molecular weight, 100-700 kDa (data not shown). This highly specific anti-TK1 antibody is unique in its ability to identify un-reduced serum TK1. This unique property allowed us to proceed and develop an immunoassay without reducing agents.

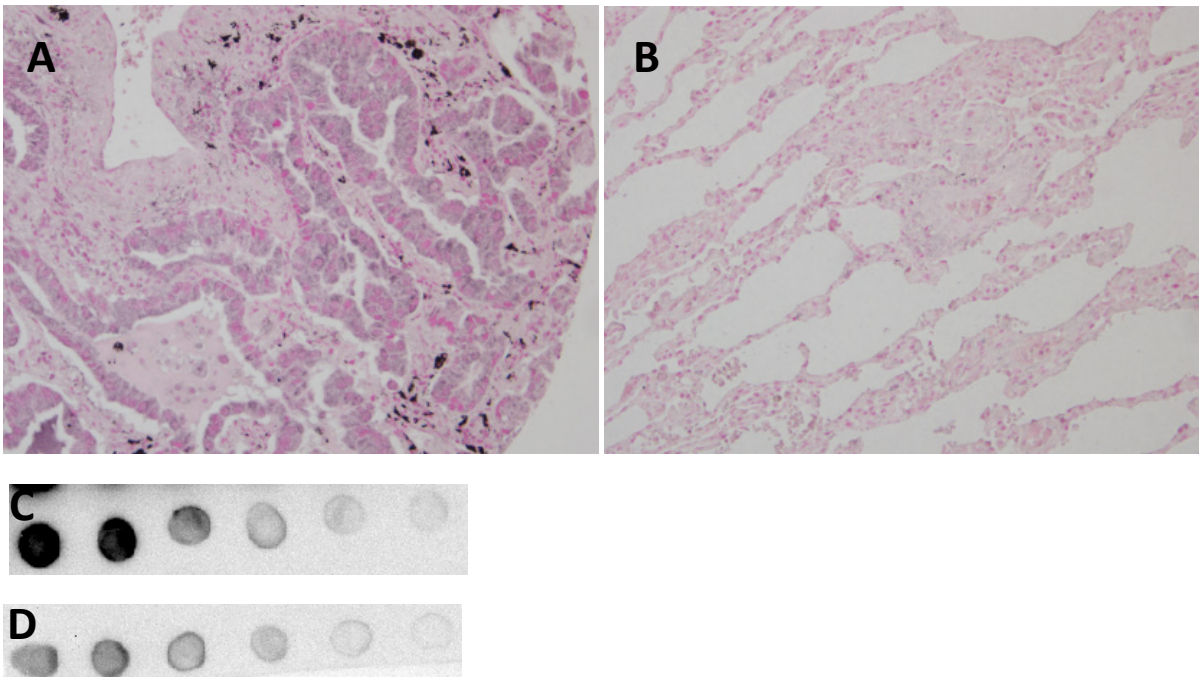


Figure 1: TK1 antibody specificity.
A) TK1 expression, detected by CB001, is elevated in squamous cell carcinoma, grade 2-3, but B) little or no TK1 expression is found in normal lung tissue. A74, a mouse monoclonal TK1 antibody, detects C) recombinant TK1, serial dilutions 707-22 ng/ml, and D) serial dilutions of native cancer serum TK1 by dot blot.

TK1 ELISA-all lung cancer cases

We used recombinant TK1 as the positive control to generate a standard curve for this novel TK1 ELISA (Figure 2). Forty pre-operable lung cancer patients, stage 1 and 2, were collected for TK1 analysis. TK1 concentration was analyzed using the TK1 ELISA while TK1 activity was determined using the traditional TK radioassay, previously optimized for human serum samples [16]. The mean (SD) of TK1 concentration in pre-operable lung cancer patients and healthy controls was 5.6 (1.32) nM and 4.5 (0.84) nM, respectively. The median (IQR) for TK1 concentration in lung cancer and healthy sera was 5.8 (5.0-6.3) and 4.4 (3.9-4.6) nM, respectively. There was a highly statistically significant difference between TK1 concentration in lung cancer patients and healthy controls as determined by the TK1 ELISA for both the Wilcoxon rank-sum and student t-test, $p<0.001$. The mean (SD) of TK1 activity as determined by the traditional TK radioassay for lung cancer and healthy controls was 8,866 (1030) cpm and 9,575 (1579) cpm, respectively. There was no statistically significant difference in TK1 activity between lung cancer and healthy sera ($p=0.69$). Since our cancer samples represent very early stage lung cancer patients, we are not surprised that the TK radioassay could not distinguish major differences in TK1 activity levels. This indicates that our novel TK1 ELISA is more sensitive and accurate than the traditional TK1 radioassay, especially for early stage lung cancer patients.

The best cut-off value for lung cancer patients (stage 1 and 2) as determined by ROC analysis was 4.9 nM. At this cut-off value, there was a highly statistically significant difference between serum TK1 concentration in lung cancer and normal controls, $p<0.0001$. The AUC was 0.828 (Figure 3). The sensitivity and specificity (95% CI) at this cut-off value was 82.4 (65.5-93.2) and 83.3 (58.6-96.4), respectively (Figure 3). The positive predictive value and negative predictive value was 90.3 (74.3-98.0) and 71.4 (47.8-88.7), respectively. The odds ratio at this same cut-off value was 23.333 (5.097-106.812), indicating that patients with a serum TK1 concentration above 4.9 nM had 23 fold increased risk of having lung cancer than those with serum TK1 levels <4.9 nM.

Two pre-operable lung cancer patients were excluded from this study because they were pretreated with chemoradiation and had less than 10% viable tumor. Since all lung cancer samples were analyzed blind, it was particularly interesting once the patient details were revealed to find an explanation for their lower than expected TK1 levels. Initially these patients were diagnosed with stage 3 lung cancer but after presurgical treatment, their levels had dropped in the range of normal and stage 1, node-negative patients. The TK1 concentrations of these two samples were 5.382 and 5.139 nM, which were excluded from the 40 pre-operable lung cancer analysis as a result of their pretreatment.

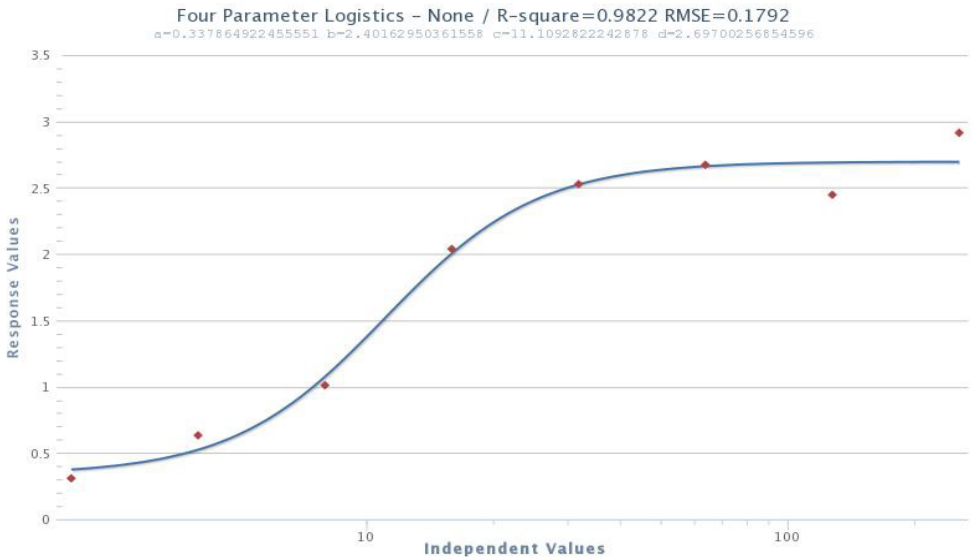


Figure 2: TK1 ELISA Standard Curve.
This standard curve was used to calculate TK1 concentrations in lung cancer and normal serum samples.

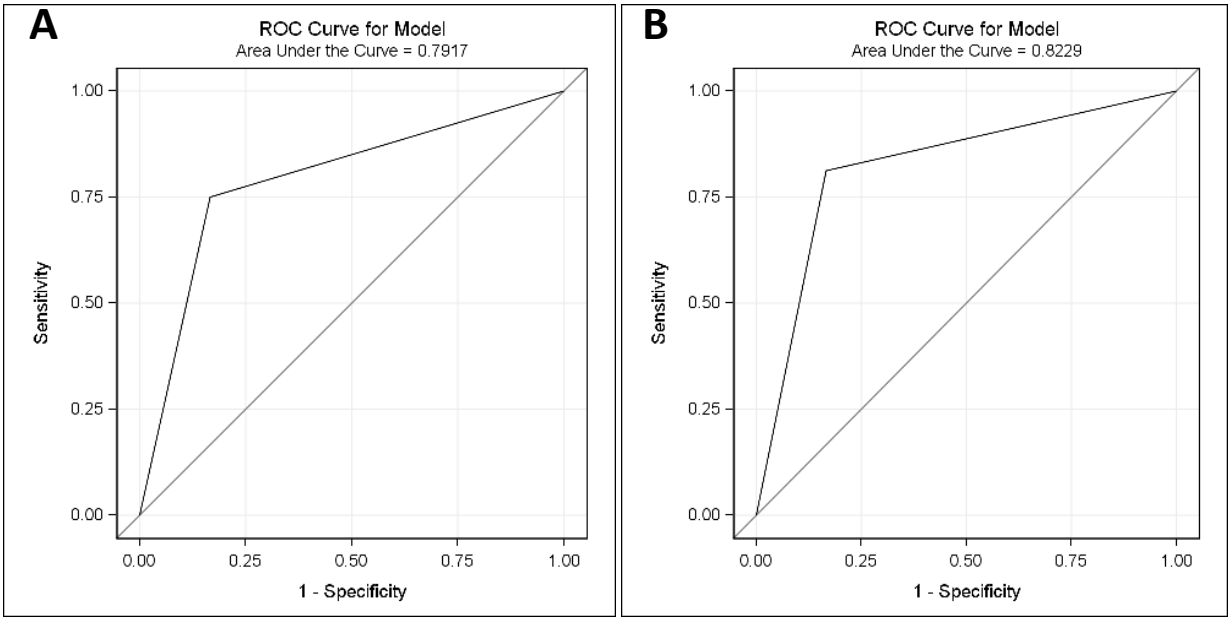
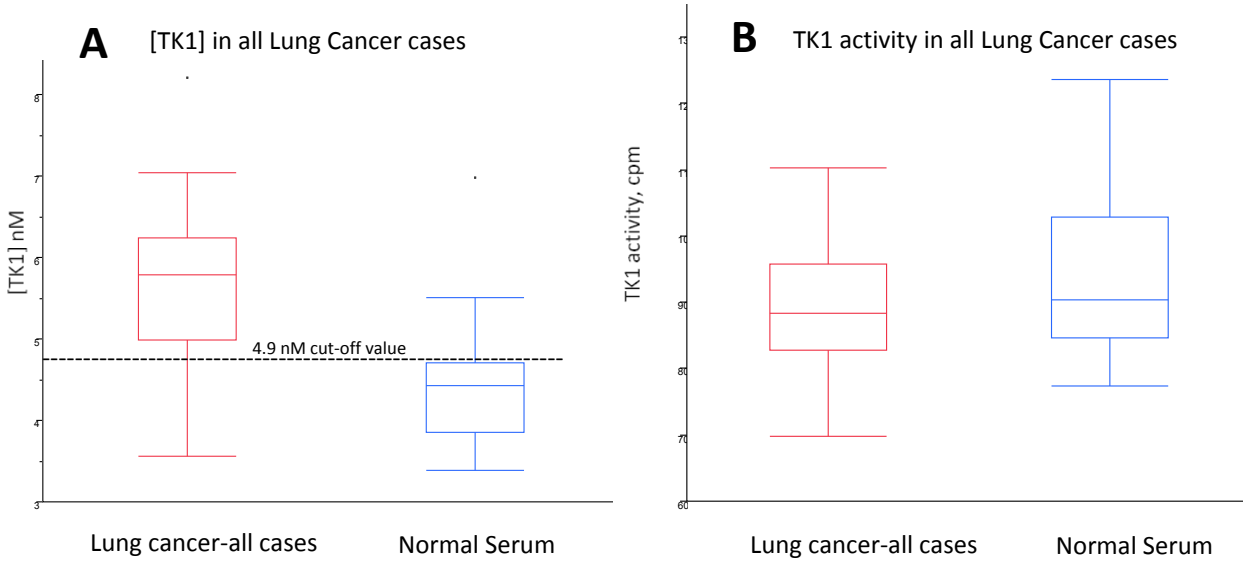


Figure 3: TK1 ELISA ROC curves.
A) The AUC for stage 1, node-negative lung cancer patients is 0.792 with a sensitivity and specificity of 75.0 and 83.3, respectively. B) The AUC for all lung cancer cases is 0.82 with a sensitivity and specificity of 82.4 and 83.3, respectively.



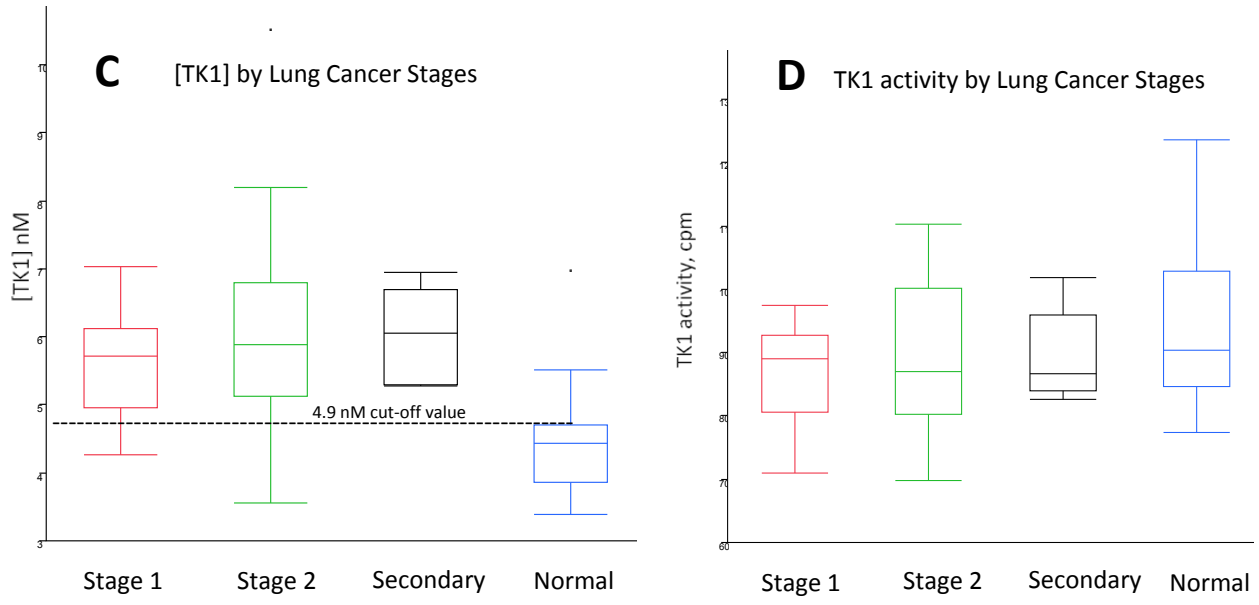


Figure 4: Box plot of TK1 distribution in lung cancer and healthy serum.
The median and interquartile range is represented by the box and center line. The maximum and minimum values, excluding outliers, are represented by the vertical lines. A) There is a highly significant difference in TK1 concentration in preoperable lung cancer serum compared with normal controls ($p<0.001$), but no significant difference in TK1 activity (B). C) There is a significant difference in TK1 concentration in stage 1, node-negative and stage 2 lung cancer patients compared to controls ($p=0.007$ and $p<0.001$, respectively). There is no significant difference in TK1 concentration between stage 1 and stage 2 lung cancer patients. D) There is no significant difference in TK1 activity among stage 1, stage 2 lung cancer and normal controls.

TK1 ELISA-stage 1, node-negative lung cancer

In an effort to determine if our novel TK1 ELISA could detect significant differences in the earliest diagnosed stage of lung cancer, stage 1, node-negative, we separated the forty lung cancer sera into stage 1 and stage 2 patients (Table 1). The mean (SD) of TK1 concentration in stage 1 (pT1) lung cancer, stage 2 lung cancer, and healthy sera was 5.3 (0.98), 6.2 (1.47) and 4.5 (0.84) nM, respectively. The median (IQR) of TK1 concentration in stage 1, stage 2 lung cancer, and healthy sera was 5.4 (4.7-6.1), 5.9 (5.2-6.8), and 4.4 (3.9-4.6) nM, respectively. According to the student t-test and Wilcoxon rank-sum test, there was a statistically significant difference in TK1 concentration between stage 1 lung cancer and healthy sera ($p=0.009$ and $p=0.007$ respectively). There was also a significant difference between stage 2 lung cancer and healthy serum according to both the t-test and rank-sum test ($p<0.001$). In either the t-test or rank-sum test, there was not a significant difference between stage 1 and stage 2 lung cancer sera ($p=0.06$ and $p=0.11$ respectively). The mean (SD) of TK1 activity in stage 1, stage 2 lung cancer, and healthy sera was 8,816 (968), 8,850 (1075), and 9,574 (1579) cpm, respectively. According to the student t-test, there was not a significant difference between any of the groups including stage 1 and stage 2, stage 1 and normal, or stage 2 and normal sera. This confirms our previous finding that the novel TK1 ELISA is more sensitive and accurate than the traditional radioassay.

The best cut-off value as determined by ROC analysis for stage 1 lung cancer and normal sera was also 4.9 nM ($p=0.002$). The AUC was 0.792 (Figure 3). The sensitivity and specificity (95% CI) was 75.0 (47.6-92.7) and 83.3 (58.6-96.4) respectively. The positive and negative predictive values were 80.0 (51.9-95.7) and 79.0 (54.4-94.0), respectively. The odds ratio (95% CI) at this cut-off value was 14.999 (2.800-80.352) indicating that patients with TK1 concentrations above 4.9 nM had 15 fold increased risk of having lung cancer, stage 1.

Of the 40 lung cancer pre-operable lung cancer sera, 6 sera were secondary lung tumors. According to the student t-test, these secondary lung tumors had significantly higher TK1 concentration levels compared with normal sera (p=0.0005) but not a significant difference in TK1 activity levels (p=0.196). There was not a significant difference in TK1 concentration or TK1 activity between primary and secondary lung tumors.

TK1 in sputum samples

Since TK1 is not a lung cancer specific marker, we sought to determine a method by which we could detect TK1 in lung cancer specifically. We obtained and blindly analyzed the TK1 activity in 17 sputum samples. Unfortunately, the sample volume was limited so only the % TK1 activity, not TK1 concentration, was determined. TK1 activity in cell extracts is often confounded by TK2 activity since ATP acts as a donor for both enzymes. However, CTP is only utilized by TK2 [17]. Therefore, we reported TK1 activity as a percentage of total TK activity, to eliminate confounding TK2 activity. This was only necessary for sputum cell extract since serum TK1 activity is not confounded by TK2 [16]. The % TK1 mean (SD) of the lung cancer sputum and normal sputum samples was 57.9% (13.8) and 32% (11.2), respectively (Figure 4). Even though only 2 normal sputum samples were available for this study, there was a statistically significant difference between lung cancer and normal sputum samples, p=0.014. A larger sample size, especially including normal sputum, is needed to confirm these preliminary results. Nevertheless, it appears that sputum TK1, possibly in connection with serum TK1, could be used for the early detection of lung cancer.

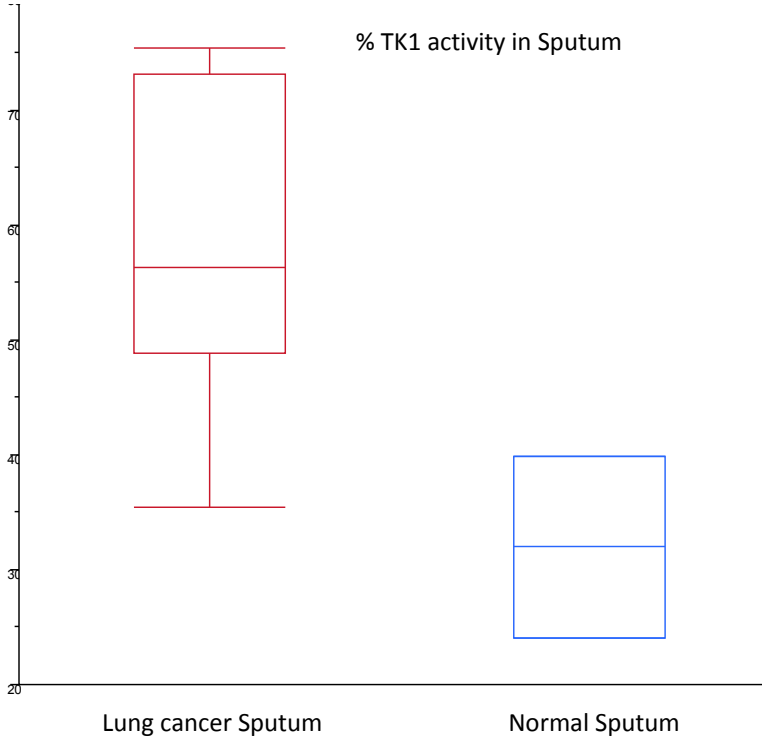


Figure 5: TK1 in Sputum.
The % TK1 activity in lung cancer sputum is significantly higher compared with normal sputum (p=0.014). Since normal sputum availability was limited, a larger sample size may be needed to confirm these preliminary results.

Discussion

Previous studies have demonstrated that TK1 is a powerful diagnostic and prognostic tool in the serum of cancer patients. Unfortunately, the clinic has not yet harnessed its potential because of challenges

associated with developing a clinical relevant method of detection. A TK1 ELISA, such as the one proposed here, overcomes limitations of using radioisotopes in the traditional TK radioassays and problems associated with specificity, sensitivity, and reproducibility. Since the ELISA platform is commonly used in the clinic, a TK1 ELISA could be adapted to other high-throughput immunoassays currently in clinical use.

This study introduces a novel TK1 ELISA which is both sensitive and specific. In addition to detecting significant differences in TK1 between advanced lung cancer and those of healthy individuals, there is a significant difference in TK1 levels of stage 1, node-negative lung cancer patients and healthy individuals. TK1 concentrations, as detected by the TK1 ELISA, show significant differences between TK1 in lung cancer and healthy individuals while TK1 activity does not. This indicates that our novel TK1 ELISA is more sensitive and accurate than traditional TK radioassays. This confirms other studies which have reported that in lung, breast, gastric, rectal, colorectal, brain cancer, leukemia, lymphoma, and hepatoma, TK1 protein concentration is more sensitive than TK1 activity [5].

In ROC analysis, the sensitivity is analogous to the true positive results or in other words, a lung cancer patient has TK1 levels above the cut-off value, as expected. A sensitivity of 100% would indicate that even asymptomatic lung cancer patients in the earliest stages of the disease, would have TK1 elevated above the cut-off value. On the other hand, specificity is analogous to true negative results. A specificity of 100% would have no false positives, such that every individual with TK1 above the cut-off value would have lung cancer. The two ROC curves representing either all lung cancer cases or only stage1, node-negative individuals had sensitivities and specificities of 82.4, 83.3 and 75.0, 83.3, respectively. A sensitivity or specificity between 90-99% could not be used for our data set since very few patients had concentrations above these cut-off values. In subsequent follow-up studies with larger sample sizes and more advanced tumors, it will likely be possible to have a sensitivity or specificity >90%. However, the AUC, sensitivity and specificity of the TK1 ELISA is comparable to other TK1 immunoassays which indicates this novel TK1 ELISA may be a reliable method of detection [14, 16, 19].

Furthermore, the positive predictive value represents the probability of lung cancer in individuals with a TK1 level above the cut-off value. On the other hand, the negative predictive value represents the probability that an individual with a TK1 level below the cut-off value does not have cancer. The positive and negative predictive values of either all lung cancer cases or only the earliest lung cancer stages were 90.3, 71.4 and 80.0, 79.0, respectively. Several studies have indicated that elevated TK1 is associated with risk for disease progression [10, 11, 13, 14, 20]. Some tumors have a lower risk of progression and a corresponding low TK1 level [21]. This is one possible explanation for why some individuals in our study with lung cancer had lower TK1 levels. This would contribute to a lower negative predictive value. A long-term follow-up study of lung cancer individuals with low preoperative TK1 levels would be needed to determine if their baseline serum TK1 levels could predict which individuals have an increased risk for disease progression. Although this study has not been conducted, similar studies have indicated that tumor TK1 expression and tumor TK1 activity can predict which tumors have an increased risk for recurrence [21-23].

One disadvantage of using a TK1 ELISA for the early detection of lung cancer is that TK1 is elevated in a variety of malignancies and is not lung cancer specific. Sputum is frequently taken from potential lung cancer patients to aid in lung cancer diagnosis. In this study, we demonstrated that TK1 activity is significantly elevated in lung cancer sputum compared to normal sputum. This indicates that perhaps screening for elevated TK1 in sputum can be an early indication of lung cancer.

Overall, it is clear that this novel TK1 ELISA is both sensitive and specific for the early detection of lung cancer. Further studies are needed to validate these exciting preliminary results. We believe this ELISA will aid in the early detection of lung cancer, the early detection of recurrent lung tumors, and monitoring a patient's response to treatment. TK1 as detected by the traditional radioassay has already proved efficacious in these areas. Therefore, we believe that this novel TK1 ELISA will similarly prove effective in monitoring a patient's response to treatment.

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Research Chapter 4: The Mechanics behind Breast Cancer Prevention

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Abstract: Cancer prevention is rapidly emerging as a major strategy to reduce cancer mortality. In the field of breast cancer significant strides have recently been made in the understanding of underlying preventative mechanisms. Currently three major strategies have been linked to an increase in breast cancer risk: obesity, lack of physical exercise, and high levels of saturated dietary fat. As a result, prevention strategies for breast cancer are usually centered on these lifestyle factors. Unfortunately, there remains controversy regarding epidemiological studies that seek to determine the benefit of these lifestyle changes. We have identified crucial mechanisms that may help clarify these conflicting studies. For example, recent reports with olive oil have demonstrated that it may influence crucial transcription factors and reduce breast tumor aggressiveness by targeting HER2. Similarly, physical exercise reduces sex hormone levels which may help protect against breast cancer. Obesity promotes tumor cell growth and cell survival through upregulation of leptin and insulin-like growth factors. This review seeks to discuss these underlying mechanisms and more behind the three major prevention strategies as a means of understanding how breast cancer can be prevented.

Introduction

Breast cancer is among the most frequently studied diseases in oncology (Siegel, Naishadham et al. 2012). In fact, breast cancer accounts for nearly 1 in 3 female cancers, and consequently is the number one most frequently diagnosed cancer in women. Fortunately, the 5-year relative survival rate of breast cancer patients has increased to 77-90% today (Siegel, Naishadham et al. 2012). However, despite recent advances in our understanding of cancer, breast cancer incidence rates have generally remained stable (Siegel, Naishadham et al. 2012). These statistics show that while there has been great improvement in detection, treatment options, and survival, there remains a great need to reduce breast cancer incidence rates through prevention.

There are several factors which have shown promise in reducing breast cancer incidence rates. Some of the most popular preventative measures relate to lifestyle choices, especially changes in diet. Despite the fact that the American Cancer Society recommends consuming a diet high in fruits and vegetables, there remains some controversy regarding the role of diet in cancer prevention (Kushi, Doyle et al. 2012). Most of this controversy comes from inconsistent results obtained from studies involving either single foods or single nutrients (Magalhaes, Peleteiro et al. 2012). The truth behind these conflicting studies is better understood by analyzing their underlying mechanisms.

Many of the lifestyle choices thought to help prevent breast cancer are either directly or indirectly related to energy balance. Energy balance is most often described as calories consumed verses those expended during physical activity (Hall, Heymsfield et al. 2012). For this reason, caloric intake and physical exercise are among the energy balance lifestyle choices which may have the greatest potential to reduce breast cancer risk. Other factors which are indirectly related to energy balance have also shown some potential with regards to breast cancer prevention. Those factors include maintaining a healthy diet of fruits and vegetables, moderate intake of red wine, and eating "good fats". In the past it has been unclear whether these factors have independent potential to reduce breast cancer rates, or whether they are simply a means of controlling overall caloric intake, another possible prevention

strategy. Rather than focusing on epidemiological studies, this review will focus on the underlying mechanisms behind breast cancer prevention to more fully understand the science behind these controversies.

Obesity

Traditionally, the most common indicator of energy balance is body mass index (BMI) which estimates body fat using an individual's height and weight. Obesity, as defined by a BMI greater than 30, is a known risk factor for a variety of diseases including cancer. This is especially true for breast cancer in which the proximity of the adipose tissue may have a direct influence on the tumor (Brown and Simpson 2012; Wang, Lehuede et al. 2012; Zhao, Sachs et al. 2012). Unfortunately, the evidence linking BMI and breast cancer appears complex. For example, obesity during pre-teen years (ages 5-10) is inversely related to breast cancer risk (Baer, Tworoger et al. 2010). On the other hand, obesity after menopause is positively related to breast cancer incidence (Carmichael and Bates 2004; Lahmann, Hoffmann et al. 2004; Cheraghi, Poorolajal et al. 2012; Phipps, Buist et al. 2012; Xu, Sun et al. 2012). Although this is fairly well established, the relationship between premenopausal obesity and breast cancer risk remains less clear. The majority of studies report that obesity, before menopause, is inversely related to premenopausal breast cancer (Carmichael and Bates 2004; Palmer, Adams-Campbell et al. 2007). However, a few studies report either no association or a positive association with breast cancer (Eng, Gammon et al. 2005; Cecchini, Costantino et al. 2012; Ogundiran, Huo et al. 2012). This complexity warrants a discussion regarding the obesity-related signaling pathways.

One of the mechanisms by which obesity is now thought to contribute to breast cancer is through the increased presence of leptin. Leptin is an adipose-tissue derived signaling molecule and is overexpressed in breast cancer, particularly in high-grade tumors (Artac and Altundag 2011). Leptin contributes to cancer proliferation through activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways as well as the JAK/STAT3 pathway (Frankenberry, Skinner et al. 2006; McCormack, Schneider et al. 2011). These pathways are often associated with tumor formation due to their involvement in cell proliferation and apoptosis. Additionally, Valle et al. reported that leptin may also increase the mitogenic effects of estrogen through the alteration of the estrogen receptor alpha and beta ratio (Valle, Sastre-Serra et al. 2011). This ratio increased as a result of chronic exposure to leptin, which also enhanced estrogen-dependent transcriptional activity and increased cell growth (Valle, Sastre-Serra et al. 2011). In addition to activating cell proliferation, leptin also poses a risk to breast cancer patients through its ability to influence epithelial to mesenchymal transition (EMT). Yan et al. demonstrated that leptin increases beta-catenin levels which are essential for promoter recruitment which leads to leptin-induced EMT and tumor formation. Beta-catenin levels are increased through leptin-induced phosphorylation of glycogen synthase kinase 3 beta (GSK3 beta) by both activated Akt and by activation of Wnt signaling through MTA1 (Yan, Avtanski et al. 2012).

Another obesity-related signaling pathway which may contribute to breast cancer is insulin-like growth factors (IGFs), especially IGF-1. Obesity is known to increase circulating insulin and IGF-1 which has been linked to tumor cell growth (Brown and Simpson 2012; D'Esposito, Passaretti et al. 2012). Consequently, it plays a major role in obesity-related breast cancer. In fact, Creighton et al. analyzed the transcription signature of primary breast tumors of obese patients compared to non-obese patient's

tumors. They reported a transcriptional signature of 662 genes in the obesity-associated tumors, most of which were involved in IGF signaling (Creighton, Sada et al. 2012). This illustrates the importance of the IGF signaling pathway in obesity-related breast cancer. Overall, IGF-1 has been linked with cell survival (anti-apoptosis) in addition to tumor cell growth (Yu and Rohan 2000; Godsland 2010). This occurs primarily through activation of PI3K/Akt and MAPK/p38 pathways (Sung, Yeon et al. 2011). D'Esposito et al. reported IGF-1's release from adipocytes can be regulated by the presence of glucose and fatty acids such as palmitate or oleate (D'Esposito, Passaretti et al. 2012). IGF-1, IGF-2, and IGF-1 receptors (IGF-1R) have also been linked to estrogen (Mawson, Lai et al. 2005). For example, IGF-2 is recognized by IGF-1R and activates both estrogen receptor-alpha and estrogen receptor-beta (Richardson, Hamilton et al. 2011). Additionally, Song et al. demonstrated that estrogen can utilize either the IGF-1R or the estrogen receptor (Song, Chen et al. 2010). These underlying mechanisms, especially their link to estrogen, may help us better understand the controversy of premenopausal obesity-related breast cancer.

Diet: Monounsaturated Fats and Olive Oil

Dietary choices and calorie reduction are lifestyle choices which may help lower the risk of breast cancer (Omodei and Fontana 2011; Nogueira, Dunlap et al. 2012). The 'Mediterranean diet' and its components have been investigated for their potential to reduce an individual's risk for cancer. The 'Mediterranean diet' includes food generally consumed within this region including nuts, fruits, vegetables, legumes, whole-wheat bread, fish, olive oil (OO) and red wine (Pauwels 2010). Within this diet, olive oil appears to reduce breast cancer risk specifically (Tsuji, Tamai et al. 2012). Other aspects of the 'Mediterranean diet' have beneficial properties and may protect against other types of cancer, but are discussed thoroughly in other reviews (Egeberg, Olsen et al. 2009; Taylor, Misra et al. 2009; Kabat, Cross et al. 2010; Alexander, Weed et al. 2011; Fortes and Boffetta 2011; Fu, Deming et al. 2011; Hardy and Tollefsbol 2011; Hauner, Janni et al. 2011; Jansen, Robinson et al. 2011; Shanmugam, Kannaiyan et al. 2011; Magalhaes, Peleteiro et al. 2012).

Olive oil has two major components which are studied in relation to breast cancer: the fatty-acid component, especially oleic acid and the antioxidant component containing polyphenols. Several studies have reported an inverse relationship between olive oil or other monounsaturated fats consumption and breast cancer (Wolk, Bergstrom et al. 1998; Thiebaut, Kipnis et al. 2007; Psaltopoulou, Kosti et al. 2011; Tsuji, Tamai et al. 2012). There are also several studies which report either no or weak association between monounsaturated fat and breast cancer (Sieri, Krogh et al. 2008; Zhang, Ho et al. 2011). Therefore, it is imperative that we understand the mechanisms by which monounsaturated fats such as olive oil influence breast cancer.

The underlying mechanism by which OO affects breast cancer is primarily through human EGFR type 2 (HER2/neu) (Colomer, Lupu et al. 2008; Menendez, Vazquez-Martin et al. 2008; Menendez, Vazquez-Martin et al. 2009). Since HER2-positive breast tumors are known to be more aggressive than their counterparts, OO specifically protects against the more aggressive breast cancer tumors while having only minimal or no effect on HER2-negative breast tumors (Colomer, Lupu et al. 2008). Early work by Menendez et al. with breast cancer and HER2 demonstrated that the tumoricidal effects of OO polyphenols are blocked when the HER2+ tyrosine activity is blocked, and OO is responsible for the depletion of the HER2 protein (Menendez, Vazquez-Martin et al. 2008). This indicated that tyrosine

kinase might play a major role in the mechanism of OO and HER2. Their follow-up study indicated that OO polyphenols did not significantly decrease tyrosine kinase activity, but they did trigger more apoptosis in HER2 over-expressing MCF10A cells. Additionally, they determined that despite the fact that OO doesn't regulate tyrosine kinase activity, the protective effects of OO specifically target breast tumors which over-express the type 1 receptor tyrosine kinase HER2 (Menendez, Vazquez-Martin et al. 2009).

The polyphenols or antioxidant component of OO is also known for its ability to help protect against DNA damage, thereby reducing the risk of breast cancer. Two of the major antioxidants that have been studied in relation to breast cancer prevention are hydroxtyrosol and tyrosol. One recent study demonstrated that hydroxtyrosol is a more potent antioxidant than tyrosol due to its ability to lower the concentration of reactive oxygen species (ROS) in MCF10A cells more compared with tyrosol (Warleta, Quesada et al. 2011). Furthermore, they also showed that hydroxtyrosol was able to prevent oxidative DNA damage in three breast cancer cell lines while tyrosol did not.

Although polyphenols represent a major component of OO, other major components of OO are also known to play a role in the protection against breast cancer. Specifically, the protective effect of certain dietary fats has been linked to regulating the amount or activity of transcription factors. This is especially true regarding OO and breast cancer. For example, Menendez et al. made the observation that oleic acid found in OO is known to repress the transcriptional activity of the HER2 gene. They also showed the HER2 promoter was essential for this repression and that oleic acid also up-regulates PEA3, a transcriptional repressor of the HER2 gene (Menendez JA 2006). Mendendez el al. later found that oleic acid also down-regulates the fatty acid synthase gene (FASN) which decreases the risk that precursor lesions will become invasive (Menendez and Lupu 2006). Overall, it appears that OO has potential to reduce the risk of breast cancer formation, especially of the more aggressive types including HER2-positive and over-expressing FASN tumors.

Although most of the work involving breast cancer prevention and OO has been *in vitro*, there is also *in vivo* evidence to support the protective effects conferred by OO. For example, Escrich et al. determined that breast cancer-induced mice that were fed a diet rich in OO showed a slight protective effect over mice that were fed a high-corn diet. Additionally, the tumors in mice fed with an OO-rich diet were less aggressive, compared to controls (Escrich, Moral et al. 2011). Furthermore, OO led to modifications in the tumors which corresponded with lower proliferation, higher apoptosis and lower DNA damage, compared to control tumors. Costa et al. demonstrated that adenocarcinomas which arose from rats fed a high-OO diet were of a lower histological grade compared with adenocarcinomas from rats fed a high-corn diet. These high-OO diet adenocarcinomas were also associated with fewer necrotic and invasive areas (Costa, Moral et al. 2004). Overall, it appears that OO as a source of monounsaturated fat has the potential to decrease both breast cancer incidence rates as well as breast cancer aggressiveness.

Physical Exercise

Physical exercise as a prevention strategy against cancer is most often associated with a means of reducing the negative effects of obesity or in other words, energy imbalance. This may be one reason why the connection between breast cancer prevention and physical exercise is less well established and understood than other preventative strategies. However, new evidence has demonstrated that physical

activity may confer breast cancer-specific protection beyond reducing obesity (Bernstein, Henderson et al. 1994; Bernstein 2009; Shin, Matthews et al. 2009; Friedenreich, Woolcott et al. 2010; Morris, Jones et al. 2010; Lynch, Neilson et al. 2011; Albrecht and Taylor 2012).

Recent evidence has indicated that physical exercise prevents breast cancer primarily through its ability to reduce sex hormone levels in women. This mechanism is crucial since estrogens promote the risk of breast cancer development due to the stimulation of mitosis and regulation of cell proliferation. Friedenreich et al. sheds further light on this mechanism as it showed that physical exercise initiated a significant decrease in estradiol and increase of sex-hormone binding globulin (SHBG), with no significant impact on levels of estrone, androstenedione, and testosterone (Friedenreich, Woolcott et al. 2010). Furthermore, Kossman et al. conducted a study involving seven healthy premenopausal women at high risk for breast cancer resulted in a beneficial reduction of estrogen (18.9%) and progesterone (23.7%) after 300 minutes of vigorous physical exercise per week for seven menstrual cycles (Kossman, Williams et al. 2011). Despite these encouraging results, there remains some controversy regarding the effect of exercise and hormone levels in premenopausal women. For instance, in the 16-week WISER study, no significant differences in sex hormones or SHBG were demonstrated between exercise and sedentary groups (Smith, Phipps et al. 2011). The results of the study involving the high-risk premenopausal women may be due to the increased length of the study, the performance of vigorous activity opposed to moderate exercise, or the doubled duration of physical activity compared with the WISER study. Nonetheless, varied results were noted. One principle behind many of these studies is the fact that an exposure to higher levels of sex hormones increases breast cancer risk. Evidence for this claim is a study by Morris et al. which correlated an increased risk of breast cancer with earlier onset of ovulation in young women who were not physically active and underwent menarche at an earlier age (Morris, Jones et al. 2010). Overall, these studies demonstrate that physical activity may play an important role in lowering breast cancer risk, through lowering the levels of sex hormones, especially as a result of lifelong physical activity.

Conclusion

Several studies have demonstrated the importance of maintaining beneficial lifestyle choices throughout an individual's lifetime to reduce breast cancer risk (Bernstein, Henderson et al. 1994; Hilakivi-Clarke, Andrade et al. 2010; Cabanes, Pastor-Barriuso et al. 2011; Escrich, Moral et al. 2011; Lynch, Neilson et al. 2011; Kark, Goldberger et al. 2012). One possible explanation for this is that the initial accumulation of driver mutations which have the potential to ultimately lead to malignancy, occur in young adults. Unfortunately, few studies address this important issue. This may be a possible explanation why some diet or physical exercise studies report insignificant correlations. Perhaps the effect of these factors on breast cancer risk was not seen due to the limited time of the study, or that the potentially beneficial agents could not reverse the effects of previous poor lifestyle choices. It may be premature to disregard these factors without long-term studies which include young adults.

Overall, there are several lifestyle factors which appear to aid in breast cancer prevention. Obesity is one factor which has shown a consistent and strong correlation with increased breast cancer risk. Dietary choices, especially the type of fat consumed, and physical exercise have shown considerable promise in reducing risk. Despite some conflicting epidemiological studies, it is clear that obesity, dietary

fat, and physical exercise each have underlying mechanisms which may help protect against breast cancer. A thorough understanding of these mechanisms will no doubt lead to the discovery of novel targets and therapies which may extend the protective effects of these lifestyle choices.

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Summary

The diagnostic and prognostic significance of TK1 has been studied and validated since the 1980's. However, unforeseen challenges arose which barred TK1 from clinical use. Recent advances have overcome these challenges which have propelled TK1 again into clinical view. In this work, we validate the diagnostic and prognostic significance of TK1 using novel TK1 antibodies. We demonstrated that our antibodies are both sensitive and specific for TK1 in malignancy and hope they will facilitate TK1's clinical use.

Using a novel TK1 monoclonal antibody (CB001), we demonstrated that TK1 elevation in breast cancer is an early event. TK1 was significantly elevated in breast carcinoma and breast hyperplasia, a pre-malignant disease, compared to normal controls. Furthermore, we demonstrated that TK1 may detect tumor cells in 'normal' tumor margins (considered normal under current pathology standards), with an increased risk of progression. Some normal tumor margins overexpressed TK1 while corresponding true normal tissue from noncancerous individuals did not express TK1. Similar results were found with a variety of malignancies including lung, prostate, colon, esophagus, stomach, liver, and kidney tissues. TK1 expression increased with increasing grade of prostate cancer. Interestingly, the prostate hyperplasia tissue in our study did not overexpress TK1. Since other studies have demonstrated that elevated TK1 is associated with risk of progression in pre-malignant tissue, perhaps the prostate hyperplasia tissue in our studies did not have an increased risk of progression. We also demonstrated that TK1 is overexpressed in true normal adrenal gland cells, germ cells of the testis, and hematopoietic cells of the bone marrow. Overall, TK1 is significantly elevated in many of the major types of malignancy and may be a useful early detection tool in these corresponding cancers.

We also demonstrated a novel TK1 immunoassay which is sensitive and specific for early stage lung cancer patients. TK1 is significantly elevated in the serum of patients with the earliest stage lung cancer (pT1N0M0), stage 2 lung cancer and all lung cancer patients in this study, compared to controls. The TK1 ELISA has a high specificity and sensitivity. TK1 is also significantly elevated in sputum which may aid in developing a lung cancer specific early detection screen. We hope this study can be applied to current high-throughput clinical applications to facilitate TK1 has a clinical screening, diagnostic, and monitoring treatment tool. In conclusion, TK1 is significantly elevated in the very early stages of the most common types of malignancy. We hope this work will continue to propel TK1 forward into clinical view in the coming years.

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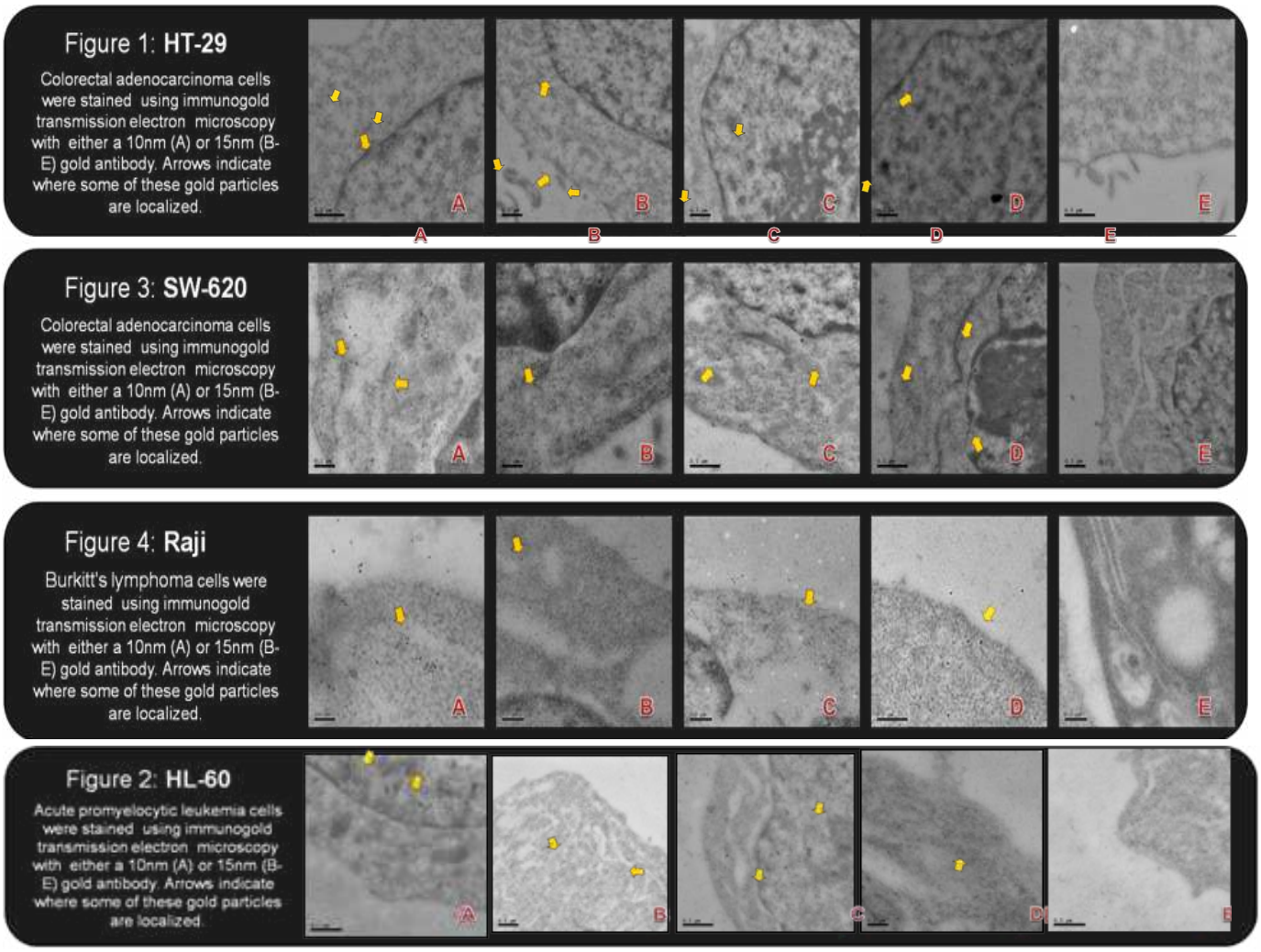
Appendix: Unpublished data

Figure 1: Localization of TK1 and other salvage pathway enzymes in human cancer cells using immunogold labeling transmission electron microscopy (TEM). A-E are as followed: TK1, APRT, dCK, HGPRT, Control.



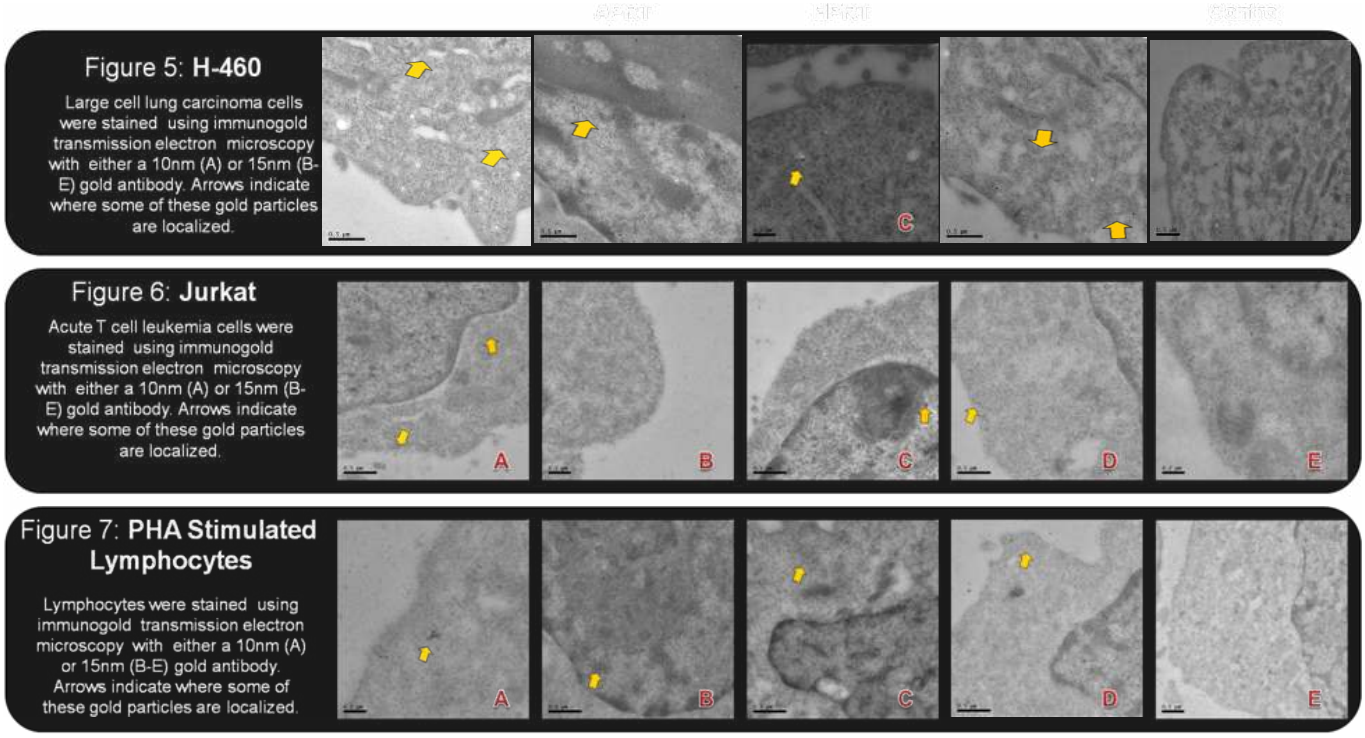
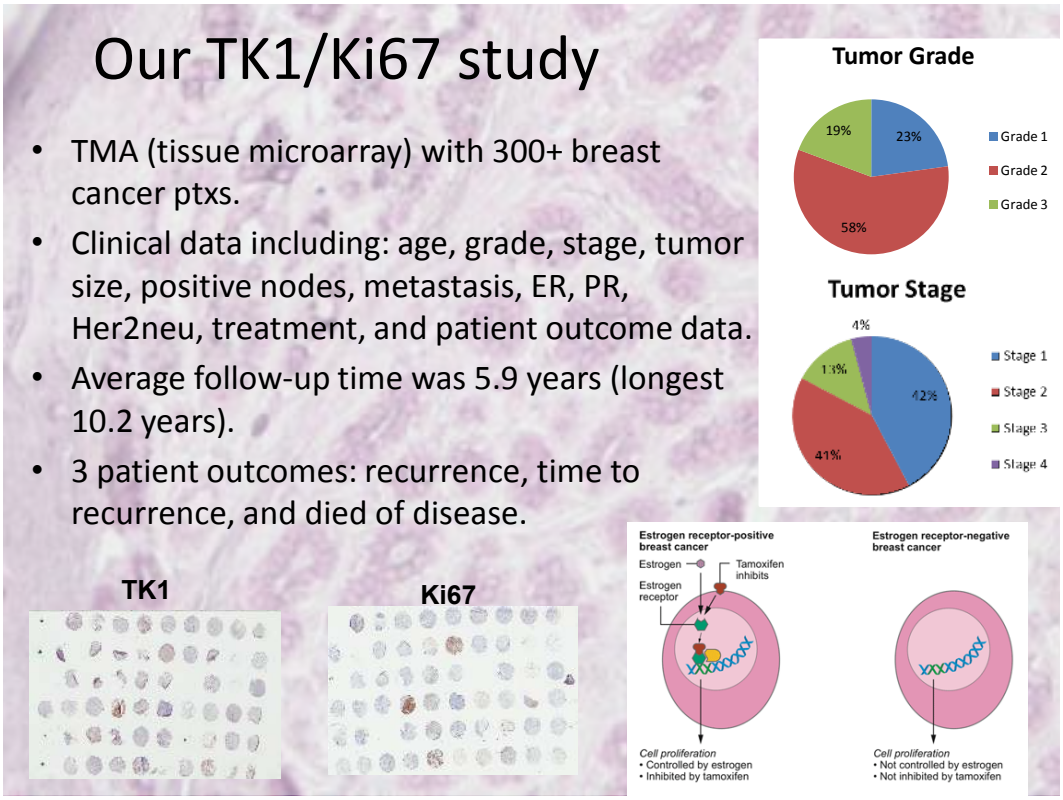
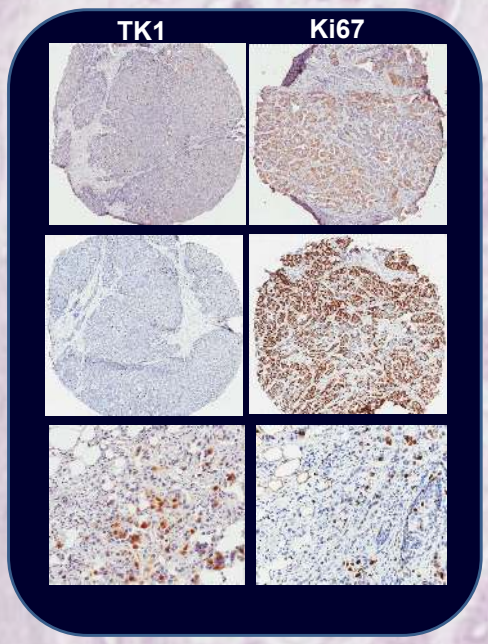
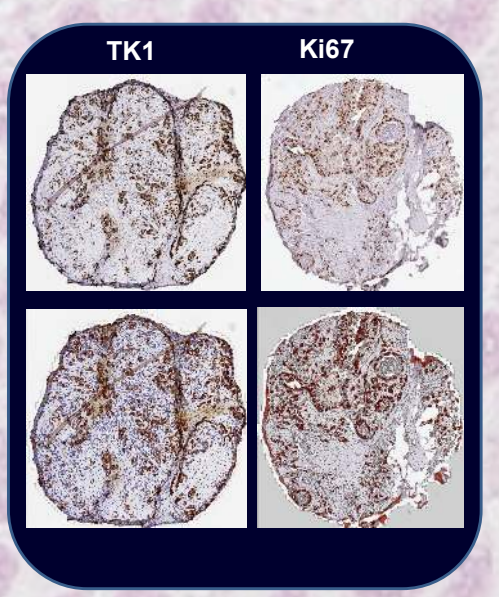


Figure 2: Prognostic significance of TK1 and Ki67 in breast cancer tumor tissue. TK1 is a better prognostic marker than Ki67. TK1 is associated with time to recurrence but Ki67 was not associate with any patient outcome.



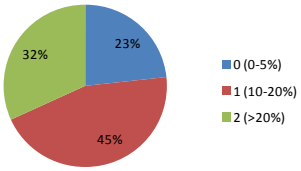
Manual vs Digital Scoring



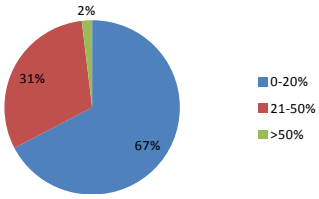


Manual vs Digital Scoring

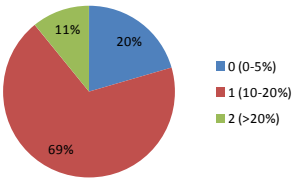
Manual TK1 scores



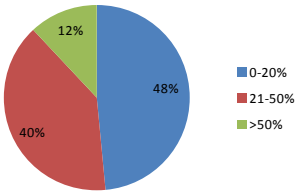
TK1 Positive Index



Manual Ki67 scores



Ki67 Positive Index



TK1 and Ki67 correlate (p<.0001)
TK1 and grade correlate (p=.003)
TK1 and ER+ correlate (p=.0073)

No Correlations found between FAS, Annexin 1, PR, Her2neu, age, diagnosis, stage, or type of treatment.

Final Predictive Models

Died of Disease:
-Positive nodes (p=.016)
-PR (p=.0108)

+1 positive node= 9.8% incr chance of cancer-related death

If PR NEG then 4.6x incr chance of cancer-related death

Recurrence:
-Positive nodes (p=.0433)
-Metastasis (p=.0262)
-Tumor size (p=.0416)

+1 positive node= 5.8% incr chance of getting recurrence.

No metastasis= 82.5% less likely of getting recurrence.

+1cm in tumor size= 10.3% incr chance of getting recurrence.

Time to Recurrence:
-Positive nodes (p=.0376)
-Metastasis (p=.0348)
-Tumor size (p=.0794)
-TK1 (p=.0627)

+1 positive node: time to recurrence is 0.04yr sooner

No Metastasis: Ave 3.2yr to recurrence

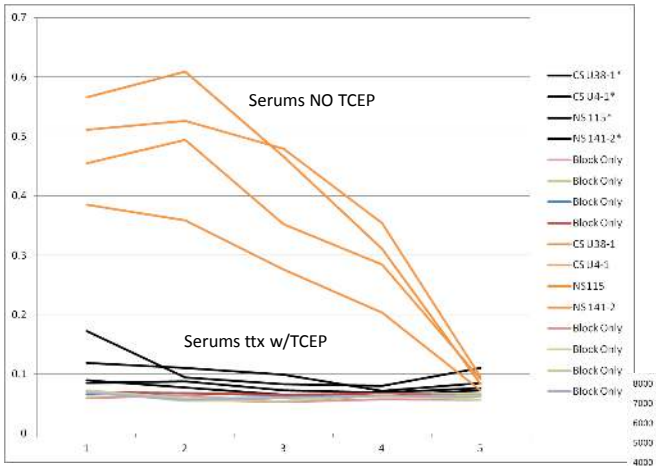
+1cm in tumor size: time to recurrence is 0.07yr sooner (baseline: 1.8yr)

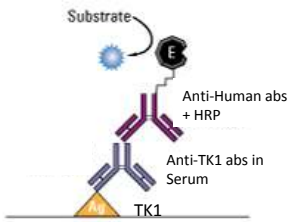
0 TK1 score: add 0.99yr before recurrence

1 TK1 score: add 0.6yr before recurrence

Figure 3: Measuring TK1 autoantibodies in the serum of cancer patients. TK1 autoantibodies are detectable by direct ELISA in the serum of cancer and healthy individuals.

Auto-antibodies to sTK1





*TK1-730kDa is inactive and will dissociate with reducing agents.

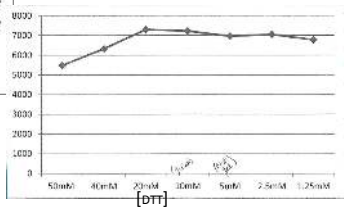


Figure 4: TK1 Sandwich ELISA. There is a significant difference in TK1 levels between lung cancer and healthy individuals. More samples are needed to confirm these findings.

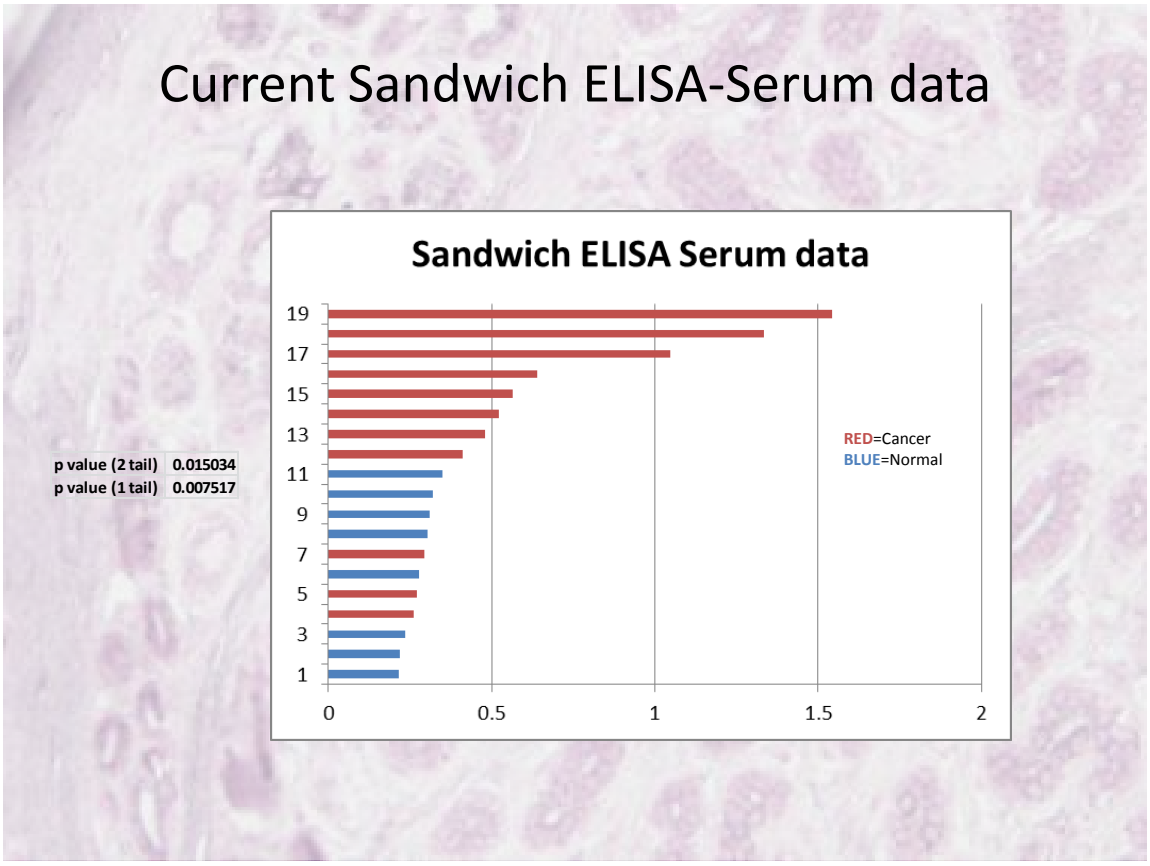


Figure 5: TK1 Competitive ELISA. In addition to creating a Direct and Sandwich ELISA, we can also detect TK1 levels using a competitive ELISA.

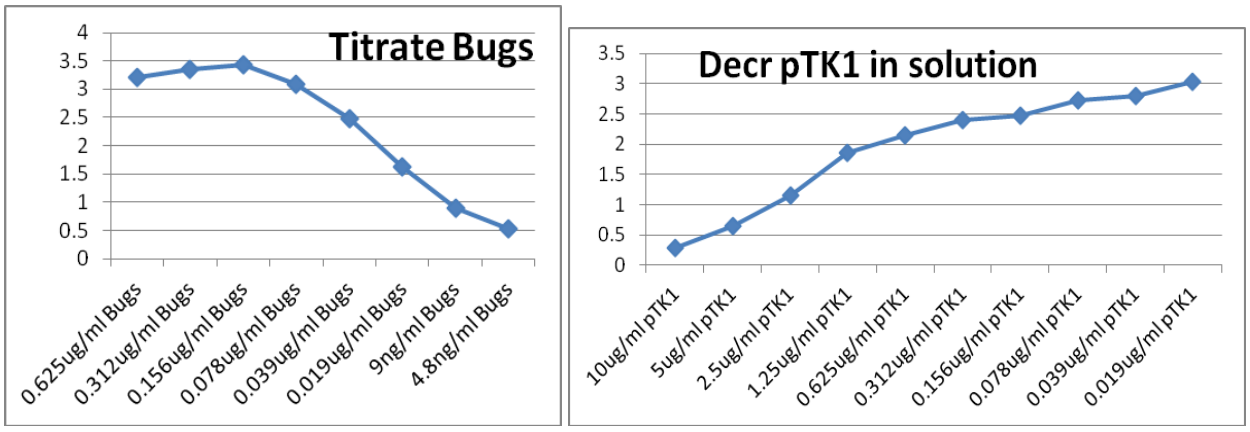
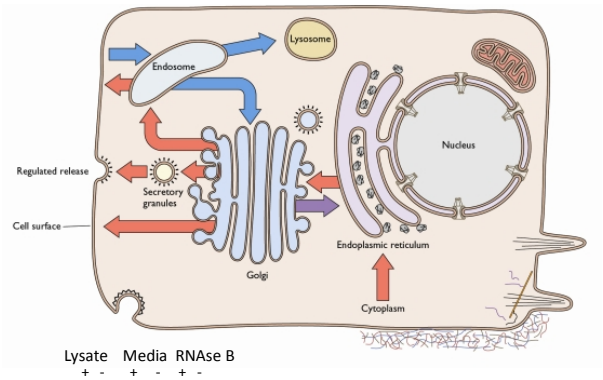
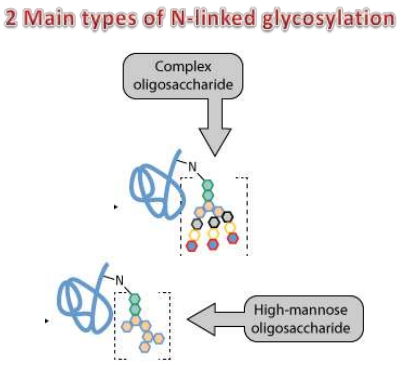


Figure 6: Serum TK1 is not heavily glycosylated according to gel mobility assay after treatment with PNGase and EndoH. Serum TK1 exists as a high molecular weight form (200-700 kDa) as detected by a native western blot.

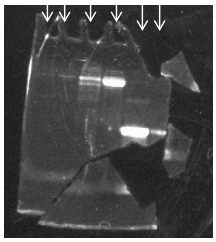
Active secretion of TK1 by cancer cells?





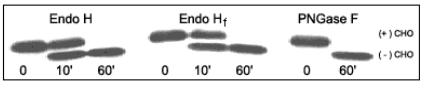
PNGase F removes all types of N-linked
Endo H removes only high-mannose and some hybrid types

Lysate Media RNAse B
+ - + - + -

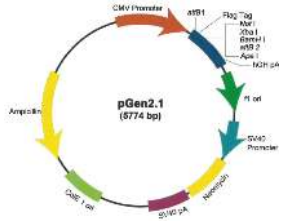


No shift for TK1

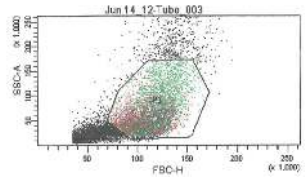
Gel Mobility Shift Assay



Alternative approaches to Mobility Shift Assay



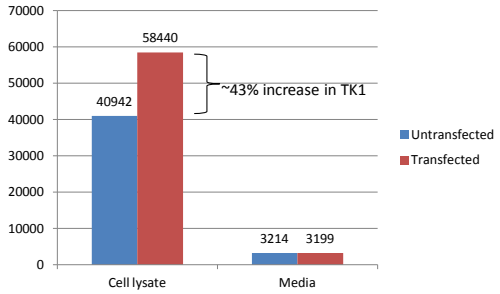
HIS-TK1 PLASMID



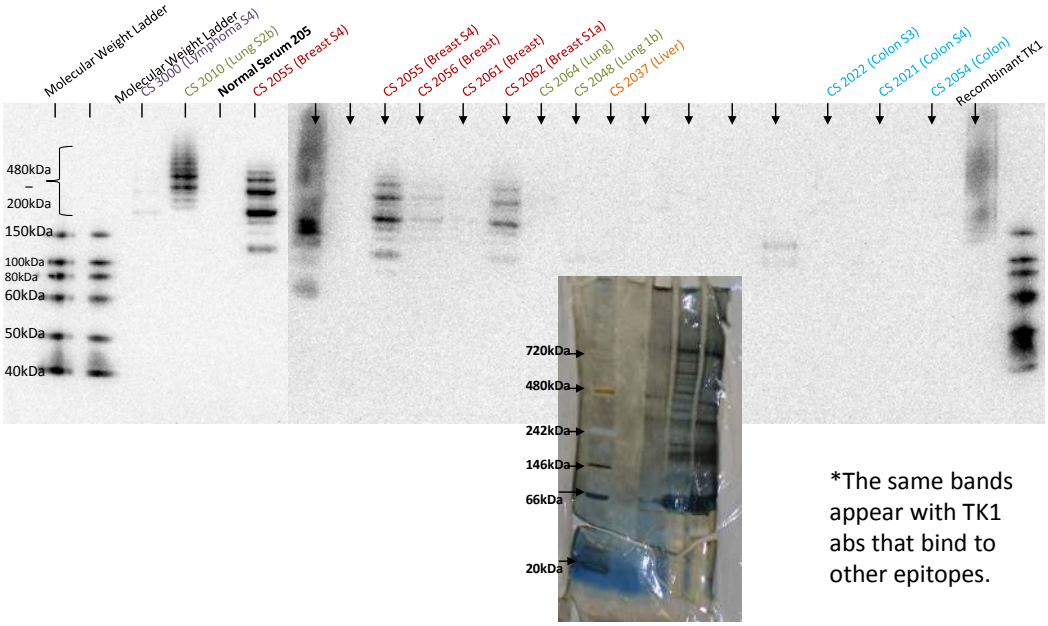
HEK293 TRANSFECTION:
28-45% TRANSFECTION RATES

ALTERNATIVE: IMMUNOPRECIPITATION

His-purification not successful due to dimerization of TK1



Visualize TK1-730kDa in cancer serum



*The same bands appear with TK1 abs that bind to other epitopes.

Detailed Materials and Methods:

IRB Approval: All patient serum was collected and consented according to IRB approval at both Brigham Young University and University of Colorado at Denver. No IRB approval was needed for purchased tumor tissue specimens from Cybrdi. All other tumor tissue specimens were collected according to IRB approval with Intermountain Healthcare.

Immunohistochemistry protocol:

A. Deparaffinization
1. Immerse array slides in xylene for 10min, repeat once in new xylene for 10 min.
2. Immerse array slides in 100% EtOH for 5 min.
3. Immerse in 95% EtOH for 5 min.
4. Immerse in 70% EtOH for 5 min.
5. Rinse array slides twice with PBS for 5 min each.

B. Antigen Retrieval (Heat-induced epitope retrieval)
1. Place deparaffinized sections in pre-boiled 0.01 M Sodium Citrate buffer (pH 6.0), keep boiling for 12 min.
2. Take out the slides and allow the slides to cool to room temperature for 20 min.

C. IHC Staining
1. The endogenous peroxidase activity is blocked at room temperature by 20 min incubation in the final 3% H2O2 in Methyl Alcohol.
2. Rinse array slides in PBS for 5 min, 3 times.

3. Apply the blocking 10% normal horse serum, incubate for 30 min at room temperature, and throw off residual fluid.
4. Apply primary antibody at the predetermined dilution of 1:100 at room temperature for 3 hours.
5. Rinse array slides three times in 1 x PBS for 5 min each.
6. Incubate array slides with a biotin-conjugated secondary anti-mouse antibody (ABC Kit, Vector Lab Inc.) at room temperature for 30 min.
7. Rinse array slides three times in 1x PBS for 5min each.
8. Incubate array slides with Streptavidin-Peroxidase (ABC Kit, Vector Lab Inc.) at room temperature for 30 min.
9. Rinse array slide 3 times in 1x PBS for 5 min each.
10. Proceed DAB Detection Kit (Vector Lab Inc.), control the degree of staining under regular microscope.
11. Wash array slides with tap water.
12. Stain and differentiate array slides in hematoxylin for 12 min.
13. Dehydration and transparency of array slides.
14. Mount cover slides on the array slides.

Western Blot (and Dot Blot) protocol:
10X Everything Buffer Recipe (2L):
60.6 g Tris base
288 g Glycine
Bring volume up to 2L with ddH2O (make sure you add some water before adding chemicals)

1X Native Running Buffer Recipe (1L)
100 mL Everything Buffer
Bring volume up to 1L with ddH2O

1X SDS-Page Running Buffer Recipe (1L)
100 mL Everything Buffer
Add 5 mL 10% SDS
Bring volume up to 1L with ddH2O

Laemmli 4X Denaturing Buffer (10mL)
4% SDS (0.8 g)
10% 2-Mercaptoethanol (1 mL)
20% glycerol (4 mL)
0.01% Bromophenol blue (0.00102 g)
0.125 M Tris HCl (2.4 mL of 1 M Tris HCl.)
2.8 mL ddH2O
Bring the pH to 6.8. Store at -20^{0}C.

Laemmli 4X Native Buffer (10mL)
20% glycerol (4 mL)
0.01% Bromophenol blue (0.00102 g)
0.125 M Tris HCl (2.4 mL of 1 M Tris HCl.)
4.6 mL ddH2O
Bring the pH to 6.8. Store at -20^{0}C.

1. Obtain your sample and mix it 1:1 with your laemmli buffer (Remember, denaturing for a SDS-PAGE and native for a native gel!!!).
2. If you are running a SDS-PAGE, place your sample in boiling water for 5 min.
3. Select your polyacrlyamide gel according to your needs.

4. Remove the bottom sticker from the gel casing (the electrophoresis will not work unless you do so!).
5. Place the gel in the running box and fill box with running buffer.
6. Carefully remove the comb by pulling straight up. DO NOT allow the comb to twist as you pull up!
7. Load your samples onto the gel (fill the gel wells with running buffer before samples).
8. Run the electrophoresis for 1 hour at 200 V (constant V) or until the blue leading bands reach the bottom of the gel.

1X Transfer Buffer (2L) (Make in Walk-In Refrigerator with Cold Water)
200 mL Everything Buffer
200 mL Methanol
10 mL 10% SDS
Add ddH2O to 2L. DON'T CHANGE THE pH! (Keep cold until use)
**For native gels, don't add the SDS.

1. While you gel has at least 30 min left, cut out a piece of PVDF membrane the size of the filter paper.
2. Briefly (<5 min) soak your membrane in methanol.
3. Transfer your membrane to transfer buffer and let equilibrate for at least 30 min.
4. When your electrophoresis is finished, place one black filter pad on the left side of the construction kit in cold transfer buffer.
5. Briefly soak one piece of filter paper by placing it on top of the black filter pad in cold transfer buffer on the left side of the construction kit.
6. Carefully break open the gel casing and place the gel on top of the filter paper.
7. Place your cassette with the black side down on the right side of the construction kit.
8. Place your gel, filter paper, and black filter pad on the black side of the cassette, with the black filter pad at the bottom.
9. Take your membrane and very carefully without touching the membrane as much as possible place it on the gel.
10. Carefully roll out any air bubbles between the membrane and the gel.
11. Briefly soak another piece of filter paper and place it on top of the membrane.
12. Briefly soak another black filter pad and place it on top of the filter paper.
13. Close the cassette and place it in the transfer box black to black and red to red (or clear to red).
14. Place an ice pack and a stirbar in the transfer box and fill with transfer buffer.
15. Place the transfer box on a stirplate and have the stirbar stir.
16. Run transfer at 50 V (constant V) for 2 hours in the back room of the refrigerator on the 7th floor (across the hallway from the autoclave room). Do not let your transfer get to hot, as it could destroy your membrane and proteins!

10X TBS (1L)
24.228 g Tris HCl
87.66 g NaCl
Fill up to 1L with ddH2O

TBS-T (1L)
100 mL 10X TBS
1 mL Tween-20
Fill up to 1L with ddH2O

5% Non Fat Dry Milk (NFDM) (50 mL)
2.5 g NFDM
Fill to 50 mL with 1X TBS

1. Carefully remove the membrane from the cassette without touching it and check for the presence of the molecular weight ladder.
2. Rinse once with TBS-T.
3. Block for 30 min with 5% NFDM.
4. Rinse 3 times with TBS-T for 5 min.
5. Prepare your 1^0 antibody (see table on following page).
6. Cut membrane and place membranes in labeled plastic bags.
a. If using small plastic bags, use 5 mL 5% NFDM per bag.
b. If using large plastic bags, use 15 mL 5% NFDM per bag.
7. Fill plastic bags with antibody/milk mixture, and carefully roll out all air bubbles.
8. Incubate your blot with 1^0 antibody overnight while shaking at 4^0 C.
9. Rinse 3 times with TBS-T for 5 min.
10. Prepare your 2^0 antibody (see specific tables below).
a. If using small plastic bags, use 5 mL 5% NFDM per bag.
b. If using large plastic bags, use 15 mL 5% NFDM per bag.
11. Fill plastic bags with antibody/milk mixture, and carefully roll out all air bubbles.
12. Incubate your blot with 2^0 antibody for at least 2 hours (or longer) while shaking at 4^0 C.
13. Rinse 5 times with TBS-T for 5 min.
14. If using a biotin-streptavidin system, incubate with your 3^0 antibody as above.
15. For HRP system (not chemilluminescent), add enough TMB to uniformly cover the membrane. Place in dark area for development for at least 20 minutes (usually longer).
16. Rinse with ddH2O and take pictures!
17. For other systems, follow the instructions of those systems.
NOTE: For chemilluminescence, double the wash times, and half the amount of 2^0 concentration (included in protocol packet).

Direct ELISA protocol
• Dilute serum in Carbonate buffer, pH 9.6, in serum dilutions starting at 1/2.
• Add 50ul diluted serum or other sample to clear 96-well plate (Thermo-Fisher).
• Incubate overnight in cold room, on shaker (70rpm, 16-17 hours).
• Allow plate to shake at same speed at room temperature for 30 minutes while Block is prepared.
• Prepare Block as followed: 2% BSA in Pierce Protein-Free T20 (PBS) Blocking Buffer (Thermo-Fisher) (i.e. 0.8g BSA in 40ml T20). Allow to sit on shaker with plate for at least 15 minutes.
• Dispense diluted serum by inverting and shaking 96-well plate.
• Using a multi-channel pipette add 300ul PBS to each well. Dispense by inverting and shaking.
• Repeat for a total of 4 washes. On the fourth wash, incubate with PBS for 4 minutes before dispensing.
• Add 300ul Block to each well and incubate on shaker at room temperature for one hour.
• Dispense Block solution. Wash with PBS as discussed above for a total of 4 washes with a 4 minute incubation during the last wash.
• Add 50ul of conjugated primary TK1 antibody (10ug/ml) diluted in Block to each well. Incubate on shaker at room temperature for one hour. All the TMB to warm to room temperature during this last hour.
• Dispense primary antibody and wash and discussed before with a total of 4 washes with a 4 minute incubation during the last wash.
• Add 50ul of room temperature Ultra-TMB (Thermo-Fisher) to each well using a multi-channel pipette. Incubate in the dark for 20 minutes at room temperature.
• Quench by adding 50ul of 2M Sulfuric Acid to each well using a multi-channel pipette.
• Read in HT Synergy Plate reader at 450nm.