AroCell TK 210 ELISA for determination of TK1 protein: age-related reference ranges and comparison with other TK1 assays

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ABSTRACT

Thymidine kinase 1 (TK1) is an enzyme involved in DNA precursor synthesis that has been used as a biomarker for prognosis and monitoring of different malignancies. In this study, we compared two immunoassays for measuring TK1 protein concentrations: the TK 210 ELISA (AroCell AB) and TK1 ELISA from Abcam. Overall, the TK 210 ELISA showed higher sensitivity than the Abcam TK1 ELISA for differentiating hematological malignancies (sensitivity of 0.77 vs 0.45) as well as for distinguishing sera of patients with solid tumors from those of apparently healthy individuals (0.61 vs 0.20). There was no significant difference in the TK1 protein levels determined with the TK 210 ELISA between different age groups from apparently healthy individuals. These results strongly indicate that the AroCell TK 210 ELISA is accurate and sensitive enough to be a valuable tool in cancer management.

METHOD SUMMARY

Thymidine kinase 1 (TK1) is an enzyme that leaks from S phase cells as a result of high cell turnover. Commercially available TK activity assays have certain limitations; to overcome these, we developed a dual monoclonal antibody-based ELISA, the AroCell TK 210 ELISA, which is commercially available. The ELISA includes a preincubation procedure with a special buffer that reduces high molecular weight complexes of serum TK1 and exposes the TK1 epitope to facilitate antibody binding. This provides a robust and convenient assay for the determination of TK1 protein concentrations in sera from patients with different malignancies.

KEYWORDS:

anti-human TK1 antibodies • AroCell TK 210 ELISA • hematological malignancies • serum thymidine kinase 1 (STK1)

Cancer is one of the leading causes of death in the world, accounting for approximately 20% of the total mortality, and annual cancer mortality is still increasing even though new chemotherapy and endocrine therapies have been developed [1]. Malignant diseases are commonly diagnosed using multiple parameters including combinations of histology, cytology, immunophenotyping, imaging and protein biomarkers. Early diagnosis of cancer is crucial for effective therapy and so increases the overall survival. Biomarkers play a crucial role in the early detection of cancer as well as in prognostication and therapy monitoring [2].

Thymidine kinase 1 (TK1) is a blood biomarker of cell proliferation that has been used for the diagnosis, prognosis and treatment monitoring of different malignancies for many years [3–5]. TK1 is an ATP-dependent pyrimidine salvage pathway enzyme involved in DNA precursor synthesis; it phosphorylates deoxythymidine to deoxythymidine monophosphate, which is further phosphorylated to di- and triphosphate and then incorporated into DNA strands [6]. TK1 expression is cell cycle dependent. Its activity increases in late G1 phase and peaks in S phase; it is then degraded by a specific ubiquitin-dependent pathway during M phase [7,8]. Several studies have reported that TK1 is a prognostic biomarker in different blood malignancies [9,10]. However, most of the commercial TK1 methods are based on enzyme activity assays that have several limitations, including the use of radioactive materials and, often, lower sensitivity in the case of solid tumors. To overcome these problems, immunoassays using anti-TK1 antibodies have been developed [11].

Clinical studies using antibody-dependent methods have demonstrated increased TK1 protein levels in sera from patients with various tumor diseases [5,12]. Furthermore, most studies have shown that TK1 protein assays are more sensitive than TK1 activity assays for prognosis and treatment monitoring of patients with these diseases [13,14]. Moreover, size exclusion chromatography analysis has shown a substantial fraction of serum TK1 that is enzymatically inactive – especially in sera from subjects with solid tumors – and which is therefore not detected by activity-based assays [15]. These findings strongly suggest that immunoassays can overcome the limitations of activity-based assays and would therefore be more suitable to routine clinical applications. Arocell AB has developed a CE-marked sandwich ELISA based on two mouse monoclonal antibodies against the C-terminal region (the TK 210 epitope) of the human

TK1 protein [16]. Recent studies have shown that the AroCell assay is as sensitive as the TK1 activity assays for distinguishing serum TK1 levels between subjects with hematological malignancies and healthy individuals, and is more sensitive for distinguishing serum TK1 levels between subjects with solid tumors and healthy subjects. Furthermore, it was shown that the TK 210 ELISA can complement tumor-specific markers such as CA 15-3 and the PSA-related antigens in diagnosis [16,17].

The two aims of this study were first to establish reference ranges for TK1 protein concentrations in sera from a large group of apparently healthy individuals and secondly to compare TK1 protein determinations made with the TK 210 ELISA and the Abcam TK1 ELISAs using sera from patients with different malignant diseases and a group of apparently healthy individuals.

Materials & methods

Study populations

Sera from apparently healthy individuals and subjects with hematological malignancies were collected at the Uppsala University Hospital, Uppsala, Sweden. Leftover sera from apparently healthy employees participating in a health surveillance project were made available to AroCell AB (n = 267). All serum samples were anonymized and only gender and age information were provided. The age range was between 26 and 70 years of age (median = 41 years; men: 123, women: 144) and the sera were divided into the following age groups: 26–35 years (inclusive), 36–50 years (inclusive) and 51–70 years (inclusive). Sixty-two serum samples from patients with hematological malignancies (HM) were collected between November 2016 and February 2019 at University Hospital, Uppsala. The HM group included native samples from patients with chronic lymphocytic leukemia (n = 29), acute myeloid leukemia (n = 11), myeloid dysplastic syndrome (n = 5), acute lymphocytic leukemia (n = 4), multiple myeloma (n = 3), chronic myeloid leukemia (n = 3), acute promyelocytic leukemia (n = 2) and others (n = 5). Sampling was approved by the Uppsala University ethical committee (2016/489) and samples were stored at -20°C until analysis. A set of sera from patients with solid tumors (n = 30; breast cancer [n = 15] and prostate cancer [n = 15]) were purchased from Promeddx (MA, USA). These samples were collected through obtaining informed consent from each individual under an IRB-approved protocol or collected as consenting donor samples from FDA-licensed/registered facilities following GMPs.

AroCell TK 210 ELISA

The AroCell TK 210 ELISA (AroCell AB, Uppsala, Sweden) was used for determination of serum TK1 protein concentration according to the manufacturer's instructions as previously described [16].

Abcam TK1 ELISA

TK1 protein concentrations were determined in a subset of serum samples from apparently healthy individuals (age 35–55 years, n = 35) and HM patients (n = 62) with the commercial Abcam TK1 ELISA (ab223595, Cambridge, UK) in accordance with the manufacturer's instructions.

TK-Liaison & ^{3}[H]-deoxythymidine (dThd) phosphorylation assays

For further comparative analysis, the TK1 enzyme activity levels were determined in another subset of sera including apparently healthy individuals (n = 30) and HM patients (n = 26) with the TK-Liaison assay and ^{3}[H]-deoxythymidine (dThd) phosphorylation assays. These sera were analyzed with all four different assays as mentioned above. The TK-Liaison assay was performed according to the manufacturer's instructions and the dThd phosphorylation assay performed as described previously [15].

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5.0 (Graph Pad Software, CA, USA). Preliminary statistical analysis using the D'Agostino and Pearson omnibus normality test revealed that the TK1 activity and TK1 protein distributions in sera from apparently healthy individuals and subjects with malignancies did not follow normal distributions. The interquartile range (IQR) represents the difference between the 75th and 25th percentiles of the group (Q3–Q1). The Mann–Whitney test was used for comparisons between the groups and the Spearman correlation coefficient (*rs*) was used to determine the correlation between TK1 protein and TK1 enzyme activity assays. The data are presented as medians. A receiver operating characteristic (ROC) analysis was used to determine the diagnostic performance of the TK 210 ELISA, the Abcam ELISA and the other assays. The level of significance was set at $p < 0.05$.

Results & discussion

Age-specific & gender-based distribution of TK1 protein levels in apparently healthy individuals

Using the AroCell TK 210 ELISA, the TK1 concentrations in sera from apparently healthy individuals (n = 264) had a range of 0.05–0.50 μg/l with a median value of 0.24 (mean ± SD = 0.24 ± 0.08 μg/l); the distribution of TK1 protein concentrations in the whole group is shown in Figure 1A. Three serum samples with TK1 concentrations of 2.2, 1.1 and 0.62 μg/l were excluded as apparent outliers. The sera were further subgrouped by age: 26–35 years (young, n = 65), 36–50 years (middle-aged, n = 142) and 51–70 years (older, n = 56), and the median TK1 protein values in young, middle-aged and older groups were 0.226, 0.241 and 0.226 μg/l, respectively. There was no significant difference in TK1 protein levels between the age groups (p = 0.76; Figure 1B) and no significant correlation between age and TK1 protein levels (p = 0.88). However, the TK1 protein levels in men were significantly higher than in women of the same age (p = 0.016;

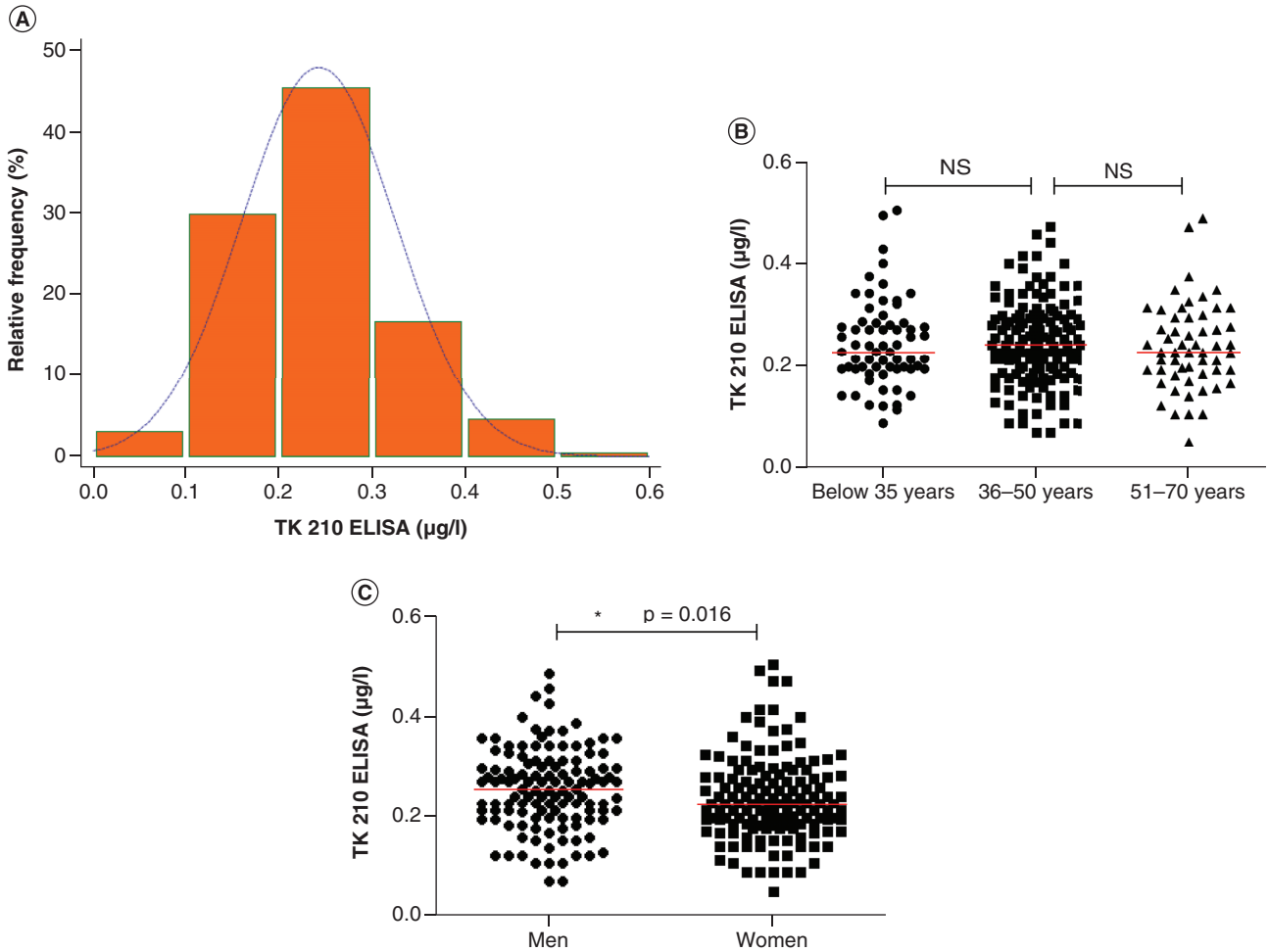

Figure 1. Serum TK1 protein levels and distribution in apparently healthy individuals. (A) Frequency distribution of TK1 protein in apparently healthy individuals (n = 264). **(B)** TK1 protein levels in different age groups: <35 years (•), 36–50 years (■) and 51–70 years (▲). **(C)** TK1 protein distribution in men (•) and women (■).
NS: No significant differences between the groups ($p < 0.05$).

Figure 1C). The 95th percentiles of TK1 protein levels in the three age groups were 0.42, 0.40 and 0.39 µg/l (IQR = 0.092, 0.104 and 0.108) respectively. The cutoff value for the whole group of apparently healthy individuals was determined by using mean and standard deviation (cutoff = mean + 2SD; TK 210 ELISA cutoff = 0.41 µg/l).

Comparison between AroCell TK 210 ELISA & Abcam TK1 ELISA

The assays were compared based on technical parameters and the instructions for use as shown in Table 1. For the clinical evaluation, subsets of sera from apparently healthy individuals (n = 35), subjects with treatment-naive HM (n = 62) and individuals with solid tumors (n = 30) were analyzed with TK 210 ELISA and Abcam TK1 ELISA.

In the apparently healthy individuals, the TK 210 ELISA concentrations showed a median of 0.22 µg/l (IQR = 0.19) with a range of 0.12–0.42 µg/l; the cutoff value of 0.42 µg/l was determined by using mean and standard deviation as above. For the sera from HM patients, the median TK1 protein value was 0.98 µg/l (IQR = 1.46) with a range of 0.14–48.5 µg/l. However, with the Abcam TK1 ELISA, the sera from apparently healthy individuals showed a median TK1 protein value of 0.5 pg/ml (IQR = 5.5), with a range of 0.0–14 pg/ml; no detectable TK1 protein was found in 50% of these sera. In the case of HM patients, the median TK1 protein concentration was 10 pg/ml (IQR = 17.2) with a range of 0–3248 pg/ml; no detectable TK1 protein was found in 10% of the HM patients.

Both the TK 210 ELISA (Figure 2A) and the Abcam TK1 ELISA (Figure 2B) were able to differentiate HM patients from apparently healthy individuals ($p < 0.0001$). Furthermore, ROC curve analysis of TK 210 ELISA showed an AUC of 0.89 ($p < 0.0001$, 95% CI = 0.82–0.95) with a sensitivity of 0.77 and a specificity of 0.95 (Figure 2C) at a cut-off value of 0.42 µg/l. The Abcam TK1 ELISA gave an AUC of 0.81 ($p < 0.0001$, 95% CI = 0.72–0.89) and the sensitivity was only 0.45 at a specificity of 0.90 using a cut-off value of 10 pg/ml (Figure 2D).

Table 1. Comparison between AroCell TK 210 ELISA and Abcam TK1 ELISA.

Assay parameter	AroCell TK 210 ELISA	Abcam TK1 ELISA
Standard series	0.5–17 μg/l	55–3500 pg/ml
Amount of sample	160 μl (duplicates)	100 μl (duplicates)
Sample type	Serum/Li-Hep plasma	Serum/Li-Hep plasma EDTA plasma/Citrate plasma
Sample dilution	1:1	1:1
Cross reactivity	No cross reactivity	Mouse and rat TK1
Internal controls	Yes (2 internal controls)	No
Assay runtime	4.45 h	1.30 h
Minimal detectable dose	0.12 μg/l	0.036 μg/l
Precision		
Intra-CV (median)	7%	10%
Inter-CV (median)	10%	11%
Wide application of TK1		
Cell cultures	Yes	Yes
Xenograft models	Yes	No

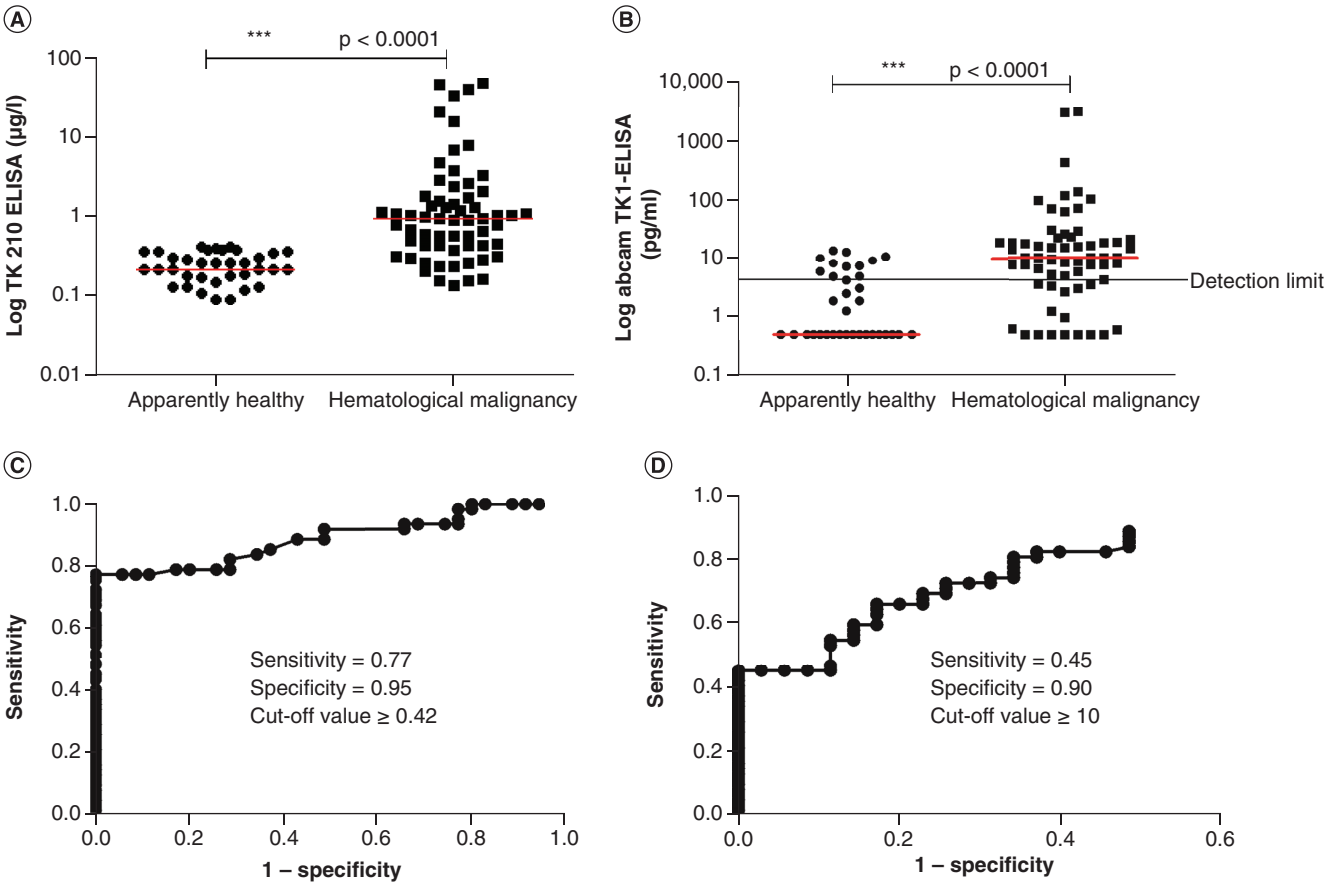

Figure 2. Serum TK1 protein levels and performance of TK1 immunoassays for hematological malignancies. Serum TK1 concentrations in subgroups of apparently healthy individuals (n = 35) (•) and hematological malignancy patients (n = 62) (■) by **(A)** AroCell TK 210 ELISA and **(B)** Abcam TK1 ELISA. **(C)** Area under ROC curve analysis for TK 210 ELISA and **(D)** Area under ROC curve analysis for Abcam TK1 ELISA in distinguishing different HM from apparently healthy individuals. The detection limit of Abcam TK1 ELISA is 6.1 pg/ml.

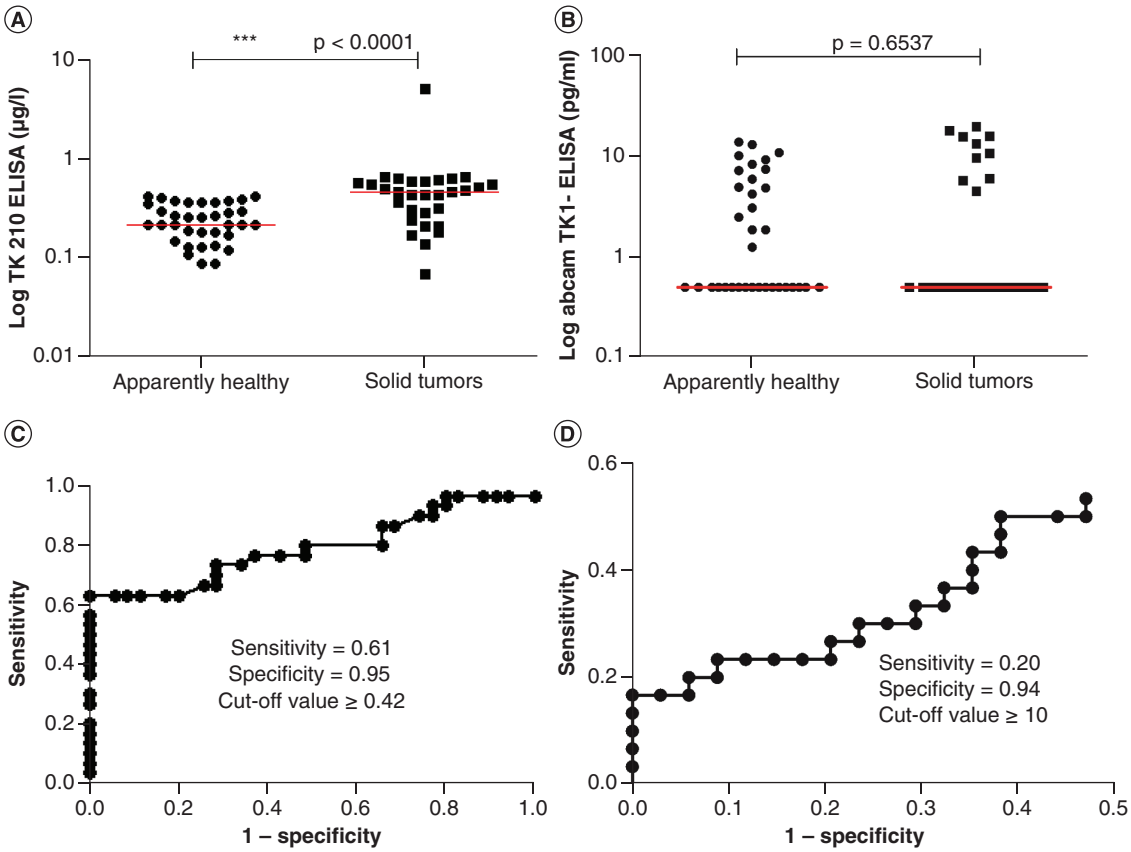

Figure 3. Serum TK1 protein levels and performance of TK1 immunoassays for solid tumors. Serum TK1 concentrations in subgroup of apparently healthy individuals (n = 35) (●) and in sera from solid tumor patients (n = 30) (■) using **(A)** AroCell TK 210 ELISA **(B)** Abcam TK1 ELISA. **(C)** ROC curve analysis for TK 210 ELISA **(D)** ROC curve analysis for Abcam TK1 ELISA.

The TK1 levels as determined by the TK 210 ELISA were significantly higher (median = 0.47 µg/l, IQR = 0.31, range 0.12–4.98 µg/l) in the sera from solid tumor patients compared with those from apparently healthy individuals ($p < 0.0001$; Figure 3A). However, 60% of the sera from solid tumor patients had no detectable TK1 using the Abcam ELISA (median = 0.5 pg/ml, IQR = 6.5, range 0.5–19.5 pg/ml) and there was no significant difference in TK1 protein levels between solid tumors and apparently healthy individuals (p = 0.65; Figure 3B). ROC curve analysis demonstrated that the TK 210 ELISA has an AUC of 0.80 ($p < 0.0001$, 95% CI 0.67–0.91; Figure 3C) and the optimal cutoff value was 0.42 µg/l, giving 0.61 sensitivity at 0.95 specificity. The ROC curve for the Abcam TK1 ELISA is shown in Figure 3D. The AUC is 0.52 (p = 0.72, 95% CI 0.38–0.66); using a cutoff value of 10 pg/ml, the sensitivity of the assay for solid tumors is 0.20 and the specificity 0.94.

Further ROC curve analysis showed a significant difference in AUC between TK 210 ELISA and Abcam TK1 ELISA (p = 0.008), as shown in Figure 4A. Overall, there was a low but significant correlation between TK 210 ELISA and Abcam TK1 ELISA (n = 125; *rs* = 0.30, p = 0.0008) (Figure 4B) after excluding the two sera with very high TK1 protein concentrations in both assays.

Correlation between TK1 assays

Another subset of sera from HM patients (n = 26) and apparently healthy individuals (n = 30) was evaluated with four different TK1 assays, two of which were based on TK1 activity measurements (TK-Liaison and dThd phosphorylation assays) and two immunoassays (TK 210 ELISA and Abcam TK1 ELISA). Significant correlations were found between TK 210 ELISA and TK-Liaison (*rs* = 0.95, $p < 0.0001$; Figure 5A), and between TK 210 ELISA and the dThd phosphorylation assay (*rs* = 0.85, $p < 0.0001$; Figure 5B). However, there was no significant correlation between the Abcam TK1 ELISA and the TK-Liaison (*rs* = 0.02, p = 0.94; Figure 5C) or the dThd phosphorylation assay (*rs* = 0.15, p = 0.47; Figure 5D).

Correlations with other blood parameters

The TK1 protein levels in HM patients, as determined with both immunoassays, were compared with other blood parameters including erythrocyte count, hematocrit, lymphocyte count and C-reactive protein (CRP). A significant correlation was observed only between TK 210 ELISA and CRP (n = 44, *rs* = 0.44; p = 0.0027) and between Abcam TK1 ELISA and CRP (n = 44, *rs* = 0.35; p = 0.018).

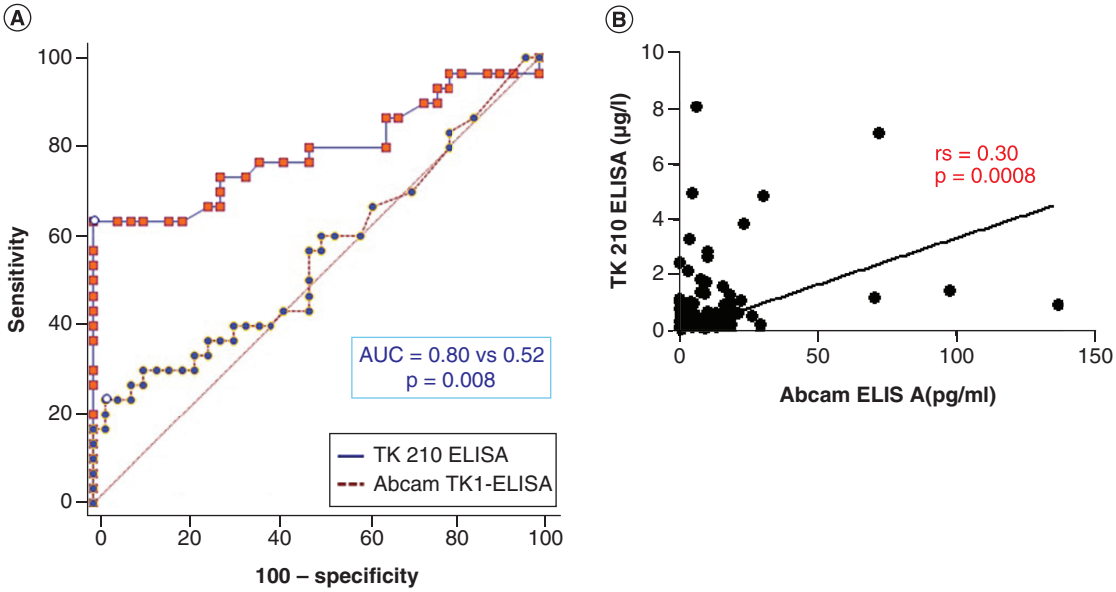

Figure 4. Performance comparison and correlation analysis of TK1 immunoassays. (A) Comparison of ROC curves between TK 210 ELISA and Abcam TK1 ELISA in differentiating solid tumors from apparently healthy individuals. (B) Correlation between Abcam TK1 ELISA and TK 210 ELISA in the subset of serum samples (n = 125) after excluding two high TK1 protein sera.

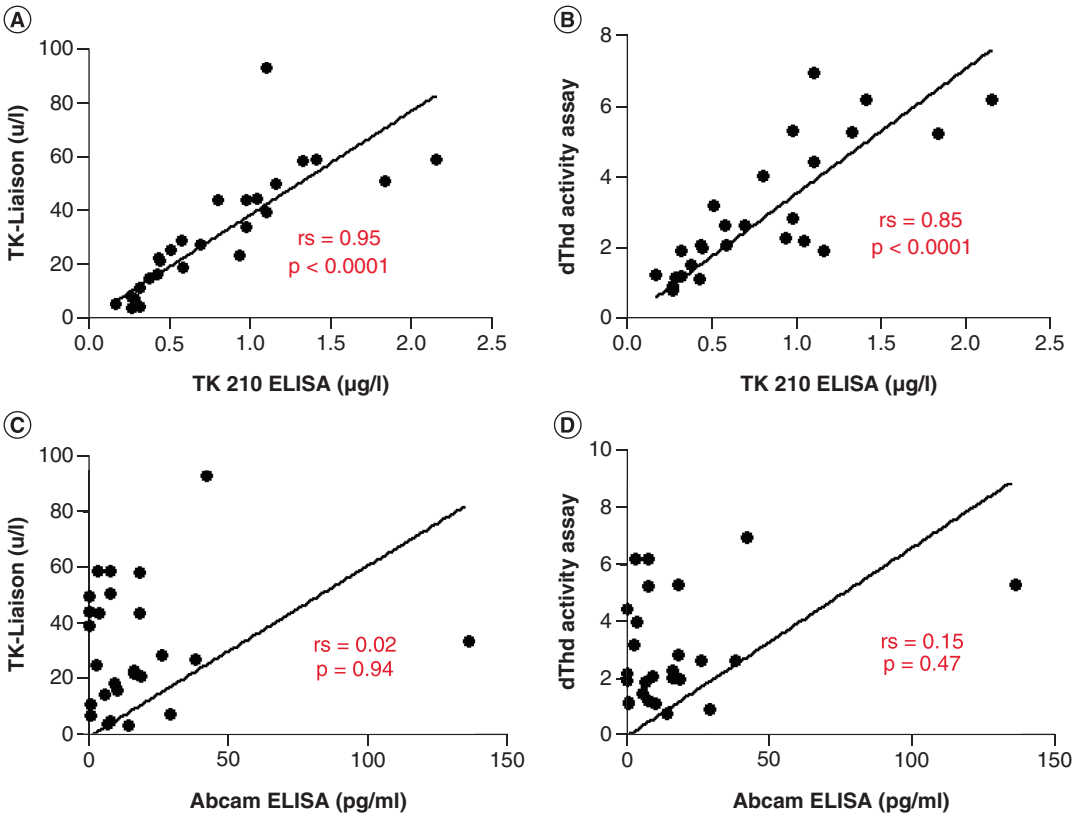

Figure 5. Correlation analysis between different TK1 determination assays. Correlations between the results with (A) TK 210 ELISA versus TK-Liaison (B) TK 210 ELISA versus dThd activity assay (C) Abcam TK1 ELISA versus TK-Liaison assay (D) Abcam TK1 ELISA versus dThd activity assay.

Uncontrolled cell proliferation is one of the hallmarks of cancer, and a biomarker that measures the proliferation rate would be valuable for tumor prognosis and therapy follow-up. TK1 is a unique blood biomarker that estimates the rate of cell proliferation and can provide valuable prognostic information – characteristics which mean it has long been used as a tumor proliferation marker for the diagnosis and prognosis of different hematological malignancies [4,9,10]. However, most of the traditional and commercial assays are based on TK1 enzyme activity and involve radioactive materials or specialized equipment. These factors have to a certain extent limited the clinical applications of TK1 as a biomarker.

The development of immunoassays based on antibodies provides an alternative approach to the measurement of TK1 activity. Several attempts have been made previously to develop commercial ELISA-based immunoassays for serum TK1 protein determinations, but none were successful [18,19]; the TK 210 ELISA is the first CE-marked standard immunoassay produced (www.arocell.com). Studies have shown that the TK 210 ELISA has high sensitivity and specificity and can complement other traditional tumor-specific biomarkers. A recent study also demonstrated that the test can be used to monitor drug effects *in vitro*, especially for those drugs that target DNA proliferation [20].

This is the first study in which a large group of apparently healthy individuals (aged 26–70 years) have been analysed with the TK 210 ELISA. There was no significant difference in the median TK1 protein levels between three different age groups (young, middle-aged and older). These results are in contrast to those of a previously published study which found that TK1 enzyme activity in healthy individuals decreased significantly with age [21]. Furthermore, TK1 protein determinations using a dot blot assay have shown a similar pattern, with serum TK1 protein concentrations increasing up to the age of 40 years and then decreasing with age [22]. The limitations in our study were the overall restricted age range was (26–70 years) and the differences in the ages of individuals included in the younger group (26–35 vs 18–35). The absence of data from subjects over 70 years of age, rather than the use of different immunoassays, may explain the differences between these two studies.

Recently, Abcam have developed several ELISA products for TK1 protein determinations. However, there is no clinical evidence available about the performance of these assays. In this study, we compare the results of the TK 210 ELISA and one of the Abcam TK1 ELISAs. The Abcam ELISA assay has the advantages of a short running time (90 min) and fewer incubation steps compared with the TK 210 ELISA (4.45 h and multiple incubations) and other Abcam ELISA products. However, according to the instructions for use, this assay cannot detect TK1 in apparently healthy individuals (based on 10 individuals); our study confirms this, as TK1 was not detectable in 50% of the apparently healthy individuals. Furthermore, the number of patient sera that were above the cutoff was significantly higher with TK 210 ELISA (45/62) than with Abcam TK1 ELISA (30/62). The ROC curve analysis further confirmed that both the AUC and the sensitivity were higher for the TK 210 ELISA compared with the Abcam TK1 ELISA. The TK 210 ELISA detected TK1 protein in all serum samples from patients with solid tumors, but this was not the case using the Abcam TK1 ELISA. Preincubation of the samples with specialized buffer and two distinctive monoclonal antibodies specific for human TK1 are the most likely reasons for the higher sensitivity and specificity of the TK 210 ELISA compared with the Abcam TK1 ELISA.

Serum TK1 exists as complexes with different molecular weights and in which the TK1-specific epitopes may be hidden. In the AroCell TK 210 ELISA procedure, preincubation of the samples with a specialized buffer breaks up these complexes and exposes the specific TK 210 epitope. This may increase the clinical sensitivity and reduce sample-to-sample variation. This is particularly important in the case of TK1 in serum from subjects with solid tumors, where much of the TK1 protein is enzymatically inactive [15]. This difference in procedure could partly explain the higher serum TK1 values found with the AroCell TK 210 ELISA compared with the Abcam assay. Furthermore, the use of the two TK 210 epitope-specific monoclonal antibodies apparently provides high specificity for the AroCell TK 210 ELISA.

Importantly, these studies demonstrated that the results from TK 210 ELISA correlate strongly with those obtained with both established TK enzyme activity-based assays. This indicates that the TK 210 ELISA measures forms of serum TK1 that are relevant for TK1 as a biomarker for cell proliferation and cell disruption. In contrast, the Abcam ELISA gave apparent serum TK1 levels that were only about 1% of those indicated by the AroCell TK 210 ELISA, and it did not show correlation with the enzyme activity assays. This indicates that the Abcam TK1 ELISA is measuring a different and probably minor fraction of serum TK1. This is important when comparing different assays and comparing results against the historic literature.

Future perspective

The development of immunoassays for TK1 protein determinations provides an alternative approach to TK1 activity assays that may enhance the clinical utility of TK1 as a biomarker. Furthermore, the compatibility of TK1 antibody assays with automated assay platforms may lead to the development of new algorithms including other cancer-related biomarkers, providing better prognosis and therapy monitoring of different malignancies.

Author contributions

K Jagarlamudi, P Venge and S Eriksson planned the study. P Venge provided the apparently healthy sera. S Holmgren analyzed the TK1 protein levels in apparently healthy individuals. K Jagarlamudi analyzed the sera from different malignancies by both assays and wrote the draft manuscript. K Levedahl and M Höglund collected the serum samples from hemtological malignancies and diagnosis. All authors read and approved the manuscript.

Ethical conduct of research

The hematological serum samples collection was approved by Uppsala University ethical board (2016/489).

Financial & competing interests disclosure

S Eriksson is an inventor of a TK1 patent licensed to DiaSorin Inc and is a shareholder in AroCell AB. K Jagarlamudi is employed by AroCell AB. We would like to thank AroCell AB for support by providing the TK 210 ELISA kits as well as direct funding of the research. Part of these results was presented as a poster at the ISOBM 2018. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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