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Review Article

IgY-Polyclonal Antibodies Raised against C-terminal 31-Peptide of Human Thymidine Kinase 1 to Detect Serum-TK1 for Risk Assessment in Colorectal Pre-carcinoma to Carcinoma Progression: A Meta-Analysis and Systematic Review

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Abstract

This study aimed at investigating whether serum thymidine kinase 1 concentration (STK1p) based on the TK1-IgY-pAb to assess the progression risk from colorectal adenoma polyp/dysplasia to colorectal carcinoma (CRC). A total of 25 publications containing patients with CRC (n=2,251), patients with colorectal polyp/dysplasia (n=1,165) and tumor-free controls (n=1,887) were analysed in the present meta-analysis. The publications were collected from PubMed, Embase, CENTRAL, CNKI, WanFang, VIP and SinoMed databases from January 1, 2009, until January 31, 2022. Articles were analysed using fixed or random effect models to calculate the mean difference. The Newcastle-Ottawa Scale was used for assessing the quality of collected studies. The meta-analysis followed the PRISMA statement. The results revealed that STK1p significantly distinguished tumor-free individuals from patients with CRC, and from patients with colorectal adenoma polyp/dysplasia ($p<0.0001$). Meanwhile, STK1p levels decreased by 34.1% within one month following surgery in CRC patients ($p<0.0001$). No significant publication bias was identified in this study. It was concluded that STK1p based on the TK1-IgY-pAb is a reliable biomarker for early detection of colorectal adenoma polyp/dysplasia, which may therefore prevent progression into colorectal carcinoma and give the patient a best chance of cure. Combining STK1p with colorectal-associated biomarkers, in addition to the determination of tumor stage and grade may therefore be of use.

Keywords: Thymidine kinase 1; STK1p; Early detection of tumor; Health screening; Meta-analysis.

Introduction

According to the Global Cancer Observatory (GCO) data for 2020, the number of newly diagnosed Colorectal Cancer (CRC) cases was about 1.93 million and the number of deaths for CRC was about 0.94 million in 2020 [1]. CRC ranks third in term of incidence and second in term of mortality worldwide [1]. The increasing burden in CRC occurs in less developed countries such as China, which means that cancer profiles in these countries are changing [2]. Population aging and lifestyle closed to Western countries might be main determinants of the incremental CRC cancer burden. The period of the progression from mutation accumulation to invasive colorectal carcinoma is about 10-15 years, which allows a time period for screening, early detection

of cancer, and removal of premalignant lesions, thus leading to an improved survival [3,4]. Currently, 5-year survival rate for CRC patients at the early stage is above 60%. However, more than 50% of CRC patients are diagnosed at the late stage. In those cases, 5-year survival rate drops to 10% [5]. Therefore, early detection of precancerous lesions or early-stage malignancies might be promising for prevention, better treatment and improved survival [6]. Reliable diagnostic, prognostic and predictive biomarkers are required to assess risk progression to carcinoma [5]. At present, the most accepted tests for CRC are fecal occult blood test, colonoscopy and sigmoidoscopy [7]. Tumor Node Metastasis (TNM) staging and carcinoembryonic antigen level are also used. However, most of these tests have been reported to be too insensitive for early tumor risk evaluation or accurate individual prognosis [8]. Tumor proliferating biomarkers with a high sensitivity and specificity are needed especially for monitoring the risk progression from neoplasm, pre-cancerous lesions to malignancies [9].

TK1 is a kinase enzyme that converts deoxythymidine to deoxythymidine monophosphate and is involved in the synthesis of DNA, and thus related to cell proliferation [9]. In this study, the serum TK1 protein concentration (STK1p) assay is based on IgY-polyclonal antibodies against C-terminal 31-mer peptide (GQPAGPDNKE NCPVP GKPGE AVAAR KLFAPQ) of human TK1 (called as TK1-IgY-pAb) [10]. The new generation STK1p concentration assay shows an area under the receiver operation characterized (ROC) curve (AUC) value of 0.96 , sensitivity=79.9% and specificity=99.7% [11] and is a more reliable assay [12] than the serum TK activity (AUC=0.71 , sensitivity=56.3% and specificity=88.4%) [13,14] and serum TK1 sandwich ELISA assay (AUC=0.90, sensitivity=50% and specificity=98%) [15].

Low STK1 levels are associated with a better prognosis for predicting recurrence and survival rate in several types of human cancer [16-19], as well as for risk assessment in pre-carcinoma to carcinoma progression. [9,20-22]. Thus, STK1p based on TK1-IgY-pAb is a useful serum-biomarker for predicting the development of malignancies and discovering early-stage tumors [9]. The Faecal immunochemical tests (FITs) are used to triage primary care patients presenting to primary care patients who had symptoms linked to colorectal cancer for referral to colonoscopy. However, combining blood test with FIT does not appear to a better discrimination for risk colorectal cancer than using FIT alone in 16,604 primary care patients [23].

In this investigation, we collected 25 colorectal studies as a meta-analysis, in order to obtain a sufficient number of cases to investigate the potential serological assay of STK1p in combine with colonoscopy for assessing progression risk from tumor-free individuals, patients with colorectal adenoma polyp/dysplasia to CRC patients, and in monitoring the effect of the surgery treatment among CRC patients.

Materials and Methods

Literature Search

A systematic literature search was conducted through the PubMed, Embase, CENTRAL, CNKI, Wanfang, VIP and SinoMed databases from January 1, 2009 until January 31, 2022, using the keywords strategy: ('thymidine kinase 1' or 'TK1') and ('colorectal' or 'colon' or 'rectal' or 'colorectum' or 'rectum') and ('cancer' or 'tumor' or 'carcinoma' or 'malignancy'). The literature search was limited to human studies. There were no language restrictions.

Inclusion and Exclusion Criteria

The searched articles were reviewed, screened, and selected by two independent reviewers strictly following the inclusion and exclusion criteria. The inclusion criteria were: (1) the STK1 concentration was measured based on TK1-IgY-pAb; (2) STK1p was measured by an enhanced chemiluminescence dot blot STK1p assay system (SSTK Ltd., Shenzhen, China) ; (3) patients with adenocarcinoma polyps were identified by clinical endoscopy and pathological diagnosis; (4) CRC patients were identified by pathological diagnosis, classified as stage I to IV and grade low to medium/high differentiation, and confirmed to have no residual tumor following surgery; (5) tumor-free individuals were used as the control group. This group was defined as tumor-free checked by different imaging, blood tests and other pathological methods.

The exclusion criteria included: (1) insufficient data; (2) using TK1 immunohistochemistry and TK1 activity methods; (3) invalid research data, which included physiological stress responses such as immunological reaction, inflammation and activation of metabolic adenosine mediators. Oxidative stress was considered a surgical stress response, together with myocardial injury, sepsis, pulmonary oedema, and kidney and liver failure, which could increase mortality; (4) tumor-free people were excluded when containing diseases associated with tumors proliferation, such as precancerous (moderate/severe types of hyperplasia of breast, prostate, gastrointestinal, cervix, liver cirrhosis, refractory anaemia); (5) people with risk diseases associated with tumors progression such as liver disease, moderate/severe fatty liver, high risk for hepatitis B, abnormal liver function, obesity and benign tumors (such as renal, thyroid); and any of the following conditions: severe cardiac disease; using any medication that could affect the STK1p levels such as exogenous hormone therapy; pregnancy; or acute illness such as inflammation/virus infection within 4 weeks. Included were people with minor type of proliferating/chronic/ non-tumor diseases.

Author/(Ref), year	Is the Definition adequate?	Representativeness of cases	Section selection of controls	Definition of controls	Comparability of cases and controls on the basis of design and analysis	Ascertainment of exposure	Exposure same method of ascertainment for cases and controls	Non-Response rate
An N *et al.*,2016	*	*	*	*	**	*	*	*
Fan X *et al.*, 2019	*	*	*	*	**	*	*	*
Huo Y *et al.*, 2015	*	*	*	*	**	*	*	*
Jiang BL *et al.*, 2018	*	*	*	*	**	*	*	*
Li PH *et al.*, 2020	*	*	*	*	**	*	*	*
Li QF, 2012	*	*	*	*	**	*	*	*
Liu X *et al.*, 2015	*	*	*	*	**	*	*	*
Li XT *et al.*,2009	*	*	*	*	**	*	*	*
Ma H *et al.*, 2021	*	*			**	*	*	*
Lu ZQ *et al.*, 2014	*	*	*	*	**	*	*	*
Ning S *et al.*, 2018	*	*	*	*	**	*	*	*
Pang ND *et al.*, 2020	*	*	*	*	**	*	*	*
Qi QF *et al.*, 2013	*	*	*	*	**	*	*	*
Shen J *et al.*, 2011	*	*	*	*	**	*	*	*
Sun HW *et al.*, 2018	*	*	*	*	**	*	*	*
Tian JG *et al.*, 2010	*	*	*	*	**	*	*	*
Weng YL, 2018	*	*	*	*	**	*	*	*
Wu HX *et al.*, 2020	*	*	*	*	**	*	*	*
Xia YZ *et al*, 2015	*	*	*	*	**	*	*	*
Xu HZ *et al.*, 2018	*	*	*	*	**	*	*	*
Zeng QH *et al.*, 2015	*	*			**	*	*	*
Zhang Y *et al.*, 2015	*	*	*	*	**	*	*	*
Zhang ZJ *et al.*, 2014	*	*	*	*	*	*	*	*
Zhu LP *et al.*, 2017	*	*	*	*	**	*	*	*
Zhu YZ *et al.*, 2015	*	*	*	*	**	*	*	*

* and ** indicates that the eight quality test options were fulfilled in each study. http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp.

Table1. Literature quality evaluation using the Newcastle-Ottawa Scale Document Quality Assessment Scale

Literature Screening and Data Extraction

Primary screening: the title and abstract of literature were carefully reviewed, and 10% of the excluded publications were randomly selected to check the concordance rate.

Secondary screening: after checking the abstracts, the full text of the publications was re-evaluated, and it was decided whether these publications should be included to the study or not, according to the criteria. Reviewer 1, 2 and 3 screened papers independently and discussed to reach an agreement; when meeting with a disagreement, the publications were rechecked by reviewer 5 and 6.

Data extraction: data were extracted from each study including: First author's name, publication year, title of publication, published journal, study population, number of samples, design type, clinical characteristics and results.

Quality Assessment

The meta-analysis followed the PRISMA guidelines. The quality of each included study was investigated using the Newcastle-Ottawa Scale (NOS). All analyses were based on previously published studies, and therefore no ethical approval and patient consent were required (Table 1).

Statistical Analysis.

RevMan 5.4 software provided by Networks of Cochrane Review Groups were used for meta-analysis. A heterogeneity test was initially performed, then a fixed or random effects model was used. A fixed effects model with an I^2 of <50% or random effects model with an I^2 of >50% was conducted to calculate the weighted mean difference and 95% confidence interval. In addition, Funnel plot and Egger's linear regression test were used to assess literature bias. For the comparison of STK1p concentration among the different groups of controls and patients, one-way analysis of variance followed by a post hoc least significant difference test was performed. SPSS version 19 was utilized for statistical analysis (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Literature search and study characteristics

As shown in figure 1, a total of 141 publications were initially identified through a search of the PubMed, Embase, CENTRAL, CNKI, WanFang, VIP and SinoMed databases. Then, 88 publications were excluded due to focusing on unrelated topics. Therefore, 53 full text articles were assessed for eligibility. Among them, 18 were excluded since another STK1 method was used instead of the SSTK dot blot ECL assay, 6 due to using different endpoints, 3 due to incomplete data, and 1 due to being a review article. Finally, 25 studies were included in this meta-analysis [24-48].

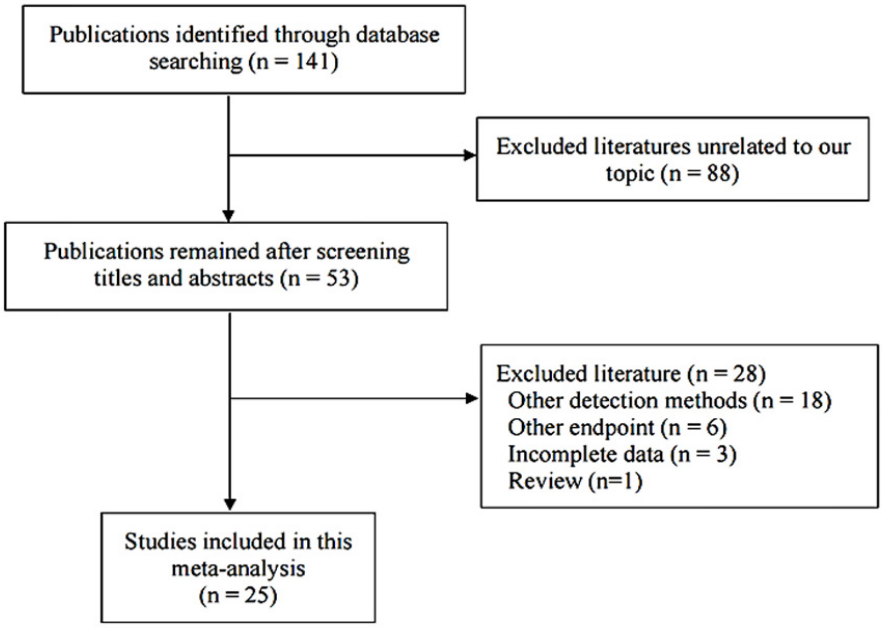


Figure 1. Flow chart of literature selection for the meta-analysis.

STK1p of tumor-free individuals compared to CRC patients.

Of the 25 publications received (Table 2), 22 were used in this comparison, including 1,887 tumor-free individuals and 1,977 patients with adenoma CRC (Figure 2A). A heterogeneity test based on the random effects model showed that the STK1p value in CRC patients was statistically higher than that in the tumor-free individuals (Figure 2A).

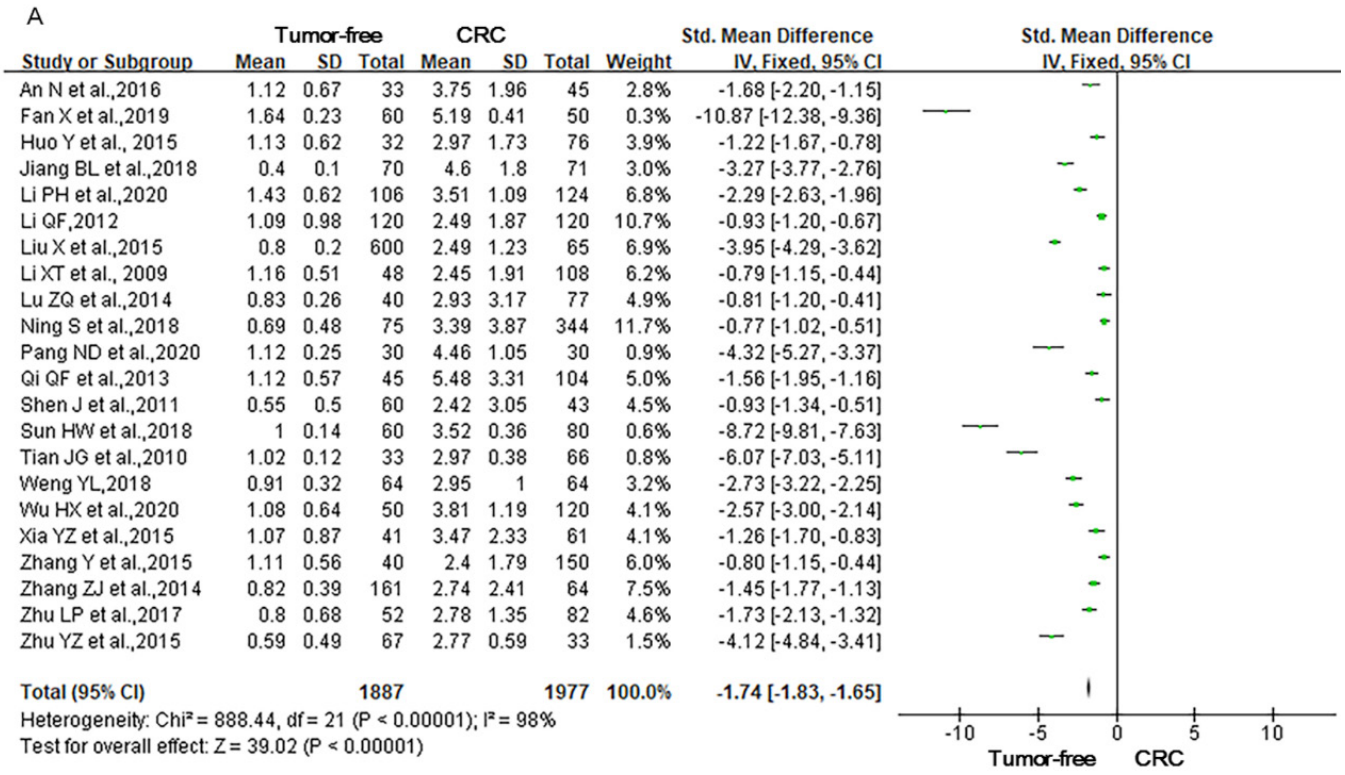


Figure 2A: Forest plots of tumor-free individuals and colorectal carcinoma patients.

Out of the 25 publications, 13 included data regarding STK1p in adenoma polyp/dysplasia and CRC (Figure 2B). There was a total of 1,165 cases in the adenoma polyp/dysplasia group and 1,178 in the CRC group. A heterogeneity test based on the random effects model showed that the STK1p values were significantly higher in CRC patients compared to the adenoma polyp/dysplasia patients.

Author/(Ref), year	Location	Tumor-free (n)	Pp./dys. (n)	CRC (n)	M (n)	F (n)	Age (years)	Clinical stage (n)				Pathological grading (n)		CRC type		Surgery	
								I	II	III	IV	Low diff.	Medium/ high diff.	Colon	Rectum	Before	After
An N *et al.*,2016	Southwest	33		45	29	16	31-76					27	18	24	21	13	13
Fan X *et al.*, 2019	Northwest	60	45	50	23	27	60-70					31	19	50			
Huo Y *et al.*, 2015	East China	32	35	76	51	25	36-83										
Jiang B L *et al.*, 2018	East China	70		71	50	21	32-82	9	26	24	12	18	53	22	49		
Li PH *et al.*, 2020	North China	106	206	124	70	54	55-71										
Li QF, 2012	Southwest	120	100	120	78	42										120	120
Liu X *et al.*, 2015	South China	600	137	65			22-67							65			
Li XT *et al.*,2009	East China	48	45	108	79	26	31-78								105		
Ma H *et al.*, 2021	North China			96			41-70									96	96
Lu ZQ *et al.*, 2014	South China	40	61	77	43	34	38-88										
Ning S *et al.*,2018	South China	75		344				132		212				177	167		
Pang ND *et al.*, 2020	South China	30	120	30	22	8	49-61										
Qi QF *et al.*, 2013	East China	45		104	58	46											
Shen J *et al.*, 2011	East China	60	50	43	28	15	30-79									43	43
Sun HW *et al.*, 2018	Northeast	60		80	50	30	24-80				80	54	26	80			
Tian JG *et al.*, 2010	East China	33		66	45	21	25-72							49	17	66	66
Wenz YL, 2018	South China	64		64	36	28	42-73	15	29	14	6				64		
Wu HX *et al.*, 2020	South China	50	65	120	69	51	67-78		73	47		39	81				
Xia YZ *et al*, 2015	East China	41		61	33	28	23-85				61	43	18	61		16	16
Xu HZ *et al.*, 2018	East China			45	23	22	24-65	19	11	8	7	15	30			45	45
Zeng QH *et al.*, 2015	East China		103	133	77	56	32-84										
Zhang Y *et al.*, 2015	North China	40	36	150	88	62	30-78	15	58	77		39	77			150	150
Zhang ZJ *et al.*, 2014	East China	161		64			35-84							35	29		
Zhu LP *et al.*, 2017	Huazhong	52	162	82	49	33	36-68	17		65		34	48		82		
Zhu YZ *et al.*, 2015	East China	67		33			16-85	7	8	8	10					33	33
Total number		**1587**	**1165**	**2251**	**1001**	**645**		**214**	**205**	**455**	**176**	**300**	**370**	**563**	**534**	**582**	**582**

Table 2. Summary of the clinical characteristics included in each selected publication（CRC：colorectal cancer; RC：rectum; M：male; F： female; diff.：differentiation；Pp./dys.: Polyp/dysplasia）.STK1p in colorectal adenoma polyp/dysplasia patients compared to CRC patients.

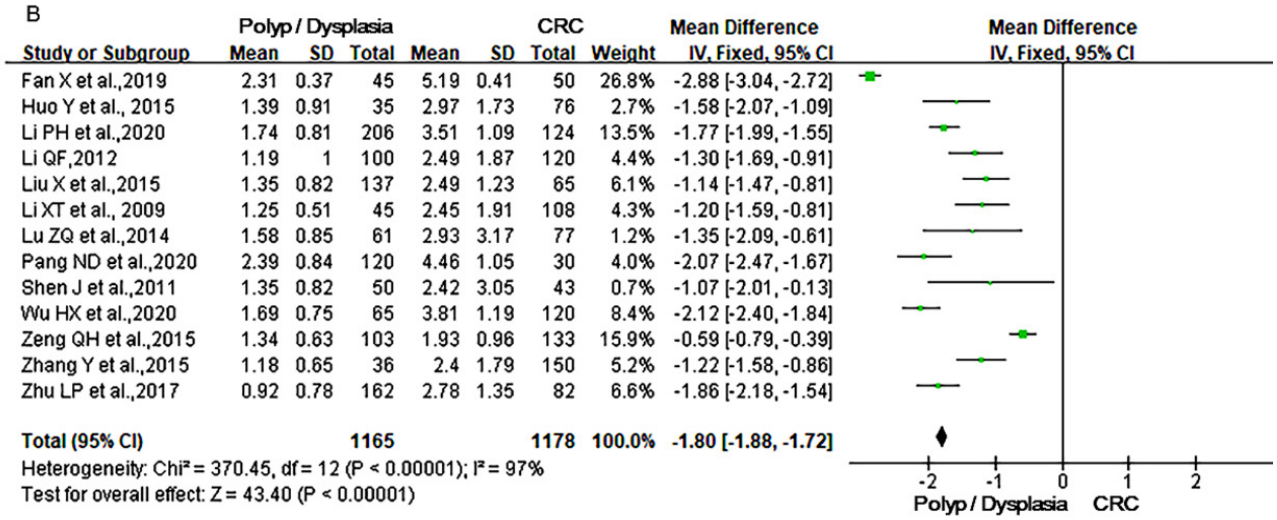


Figure 2B: Forest plots of colorectal adenoma polyp/dysplasia patients and colorectal carcinoma patients.

STK1p of tumor-free individuals compared to colorectal* adenoma polyp/dysplasia *patients.

In this comparison, 1,238 tumor-free individuals and 1,062 colorectal adenoma polyp/dysplasia patients from 12 studies were used. Based on a random effects model, the heterogeneity test showed that the STK1p value in tumor-free individuals was significantly lower than that in adenoma polyp/dysplasia patients (Figure 2C).

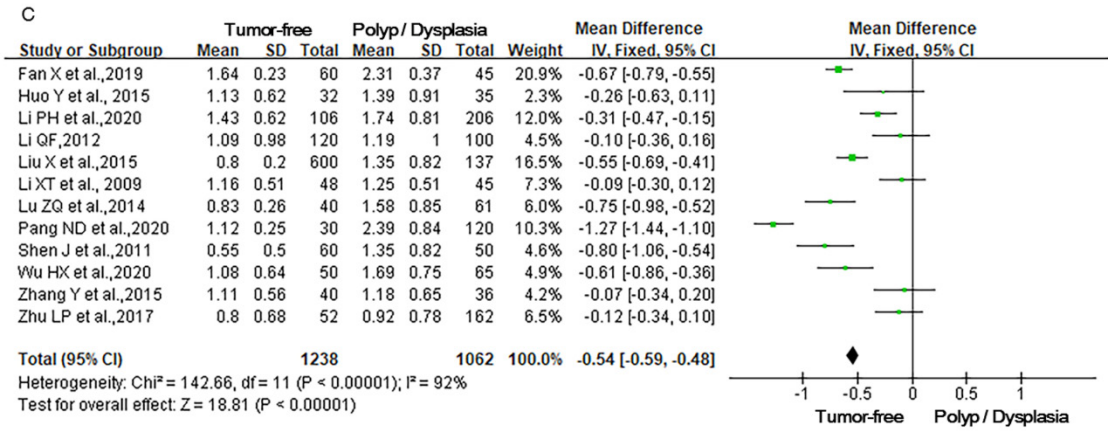


Figure 2C: Forest plots of tumor-free individuals compared to colorectal adenoma polyp/dysplasia patients.

STK1p level before and after surgery in CRC patients.

Of the 25 studies, 9 publications included data regarding the STK1p level before and one month after surgery. A heterogeneity test, based on the random effects model, showed that the STK1p value in post-surgery patients was significantly lower (34.1%) than STK1p level in pre-surgery group (Figure 2D).

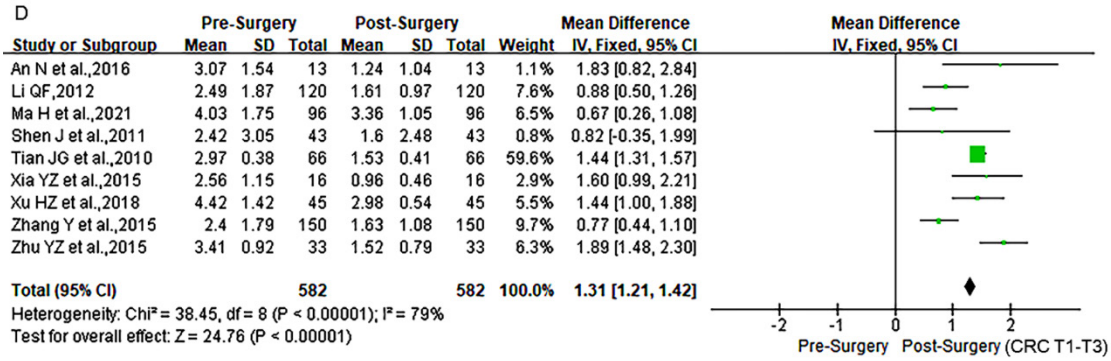


Figure 2D: Forest plots of colorectal carcinoma patients before and after surgery treatment. Green diamond indicates the mean value. SD: Standard deviation; CI: Confidence interval; IV: Inverse variance.

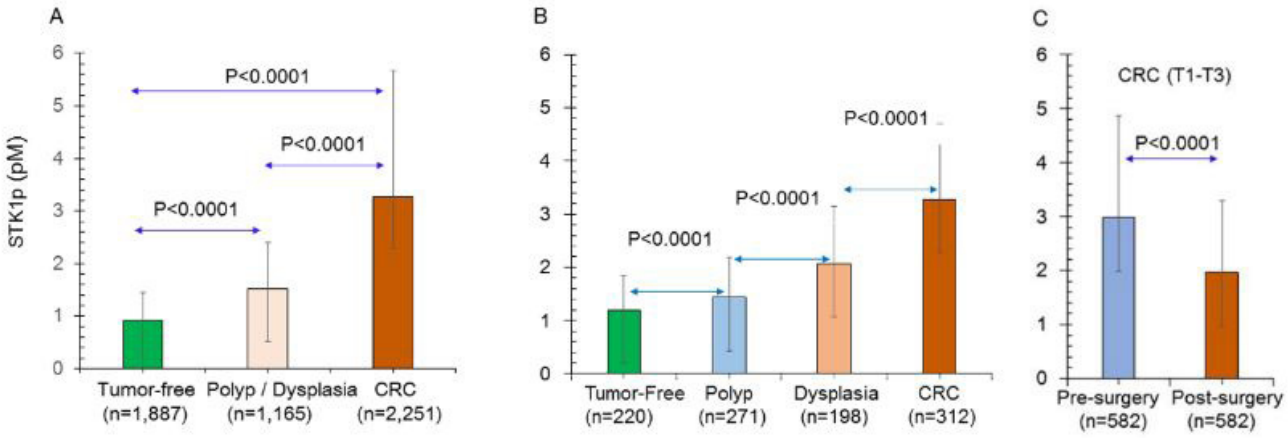
STK1p values in different populations.

The STK1p values were significantly different between tumor-free individuals (n=1,887), colorectal adenoma polyp/dysplasia patients (n=1,165), and adenoma CRC patients (n=2,251). The level of STK1p increased significantly in the following manner: tumor-free<adenoma polyp/dysplasia<carcinoma (p<0.0001) (Figure 3A).

Meanwhile, in this meta-analysis, the patients were further sub-grouped into two different categories. One was colorectal adenoma polyp group; the other was colorectal adenoma dysplasia patients defined as pre-cancer group. The tumor-free group, the polyp group and the carcinoma groups were based on four publications, respectively, while the dysplasia group was based on 3 publications. As shown in (figure 3B), the STK1p level of the CRC patients was significantly higher than the tumor-free controls and the adenoma polyp/dysplasia patients. The STK1p level increased significantly in the following manner: tumor-free<polyp<dysplasia<carcinoma.

STK1p values before and after surgery.

Among the CRC patients, the STK1p value of the post-surgery patients decreased by 34.1% one month after surgery compared to their value before surgery (Figure 3C).



Funnel plots and Egger's test

Funnel plots were conducted to evaluate the degree of bias in a graphic way. (Figure 4A-D) show a high degree of symmetry in the four groups studied (tumor free, adenoma polyp/dysplasia, CRC, pre-and post-surgery), indicating low degree of bias. Running the Egger's test further confirms no significant publication bias was identified in this meta-analysis (all p-values>0.05) (Table 3).

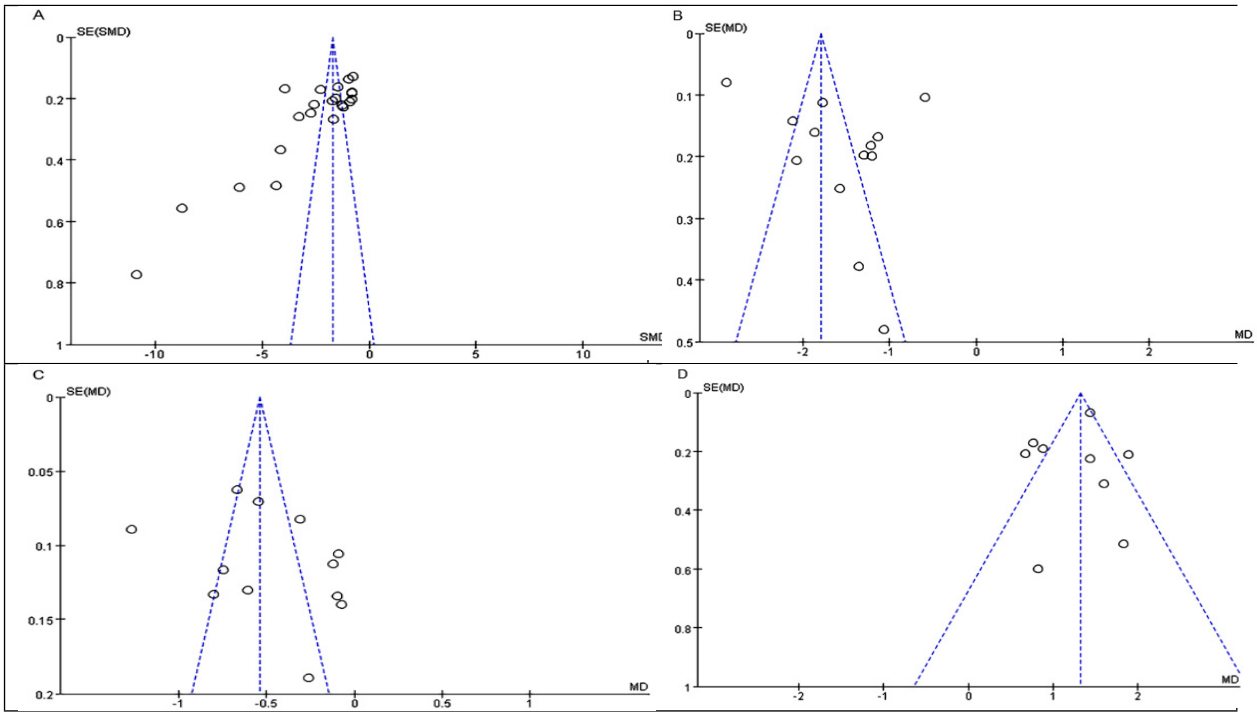


Figure 4A-D: (A) Funnel plot of tumor-free controls and colorectal carcinoma patients. **(B)** Funnel plots of colorectal adenoma polyp/dysplasia patients and colorectal carcinoma patients. **(C)** Funnel plots of tumor-free controls and colorectal adenoma polyp/dysplasia patients. **(D)** Funnel plots of colorectal carcinoma patients before and after surgery treatment.

	Coefficient	Standard error	T value	P value	95% CI
Tumor-free vs. CRC					
Bias	-.4321969	1.774517	-0.24	0.810	-4.133775 ~ 3.269381
Neoplasm vs. CRC					
Bias	1.953219	1.786894	1.09	0.298	-1.979708 ~ 5.886146
Tumor-free vs. adenoma polyp/dysplasia					
Bias	3.707167	3.254668	1.14	0.281	-3.544684 ~ 10.95902
Pre- vs. Post- surgery					
Bias	0.3391641	1.262594	0.27	0.796	-2.646397 ~ 3.324725

Table 3. Egger's tests for the assessment of publication bias.

Discussion

Meta-analysis is a statistical analysis that combines the results of multiple studies [49]. In this study, a meta-analysis was conducted according to the defined inclusion and exclusion criteria. No significant bias was found. It was also found that STK1p was able to distinguish between tumor-free, adenoma polyp/dysplasia and CRC patients. STK1p could be suggested to monitor the response of the surgery treatment in CRC patients. Thus, STK1p is a reliable biomarker for risk assessment from colorectal adenoma polyp/dysplasia and progression to carcinoma, and a useful follow-up tool for surgery.

Since cell proliferation is an important parameter in prognosis of cancer patients, we decided to use TK1, a compound closely related to DNA synthesis and thus to proliferation [11]. The STK1p was determined by an assay with high sensitivity (0.80) and specificity (0.99) and with an AUC value of 0.96 of receiver operation characteristic (ROC) statistical analysis. The STK1p assay is the most sensitive assay for TK1 in serum on the market today [11]. The mean value of STK1p in the adenoma polyp/dysplasia patients was 1.65-fold higher than that in tumor-free individuals, but significantly lower than that in CRC patients (2.13-fold) [50]. Although the mean STK1p value in adenoma polyp/dysplasia patients was higher than that in tumor-free individuals, there was a deviation, with both low and high individual STK1p values. Based on a health screening study (n=35,365) [11], patients with a STK1p value of >2.0pM were found to have a 3-5 times higher risk to develop malignancy. It was also concluded that the risk for developing colorectal malignancies should be higher in neoplasm patients with a STK1p value of >2.0pM. On the other hand, neoplasm tumor patients with STK1p values of <2.0 pM should have a low risk of developing CRC.

CRC is a heterogeneous disease, from health mucosa arising from polyp benign growth to dysplasia by accumulation of genetic mutations and to the end of carcinogenesis [51], for a long-term of 10-30 years (figure 5).

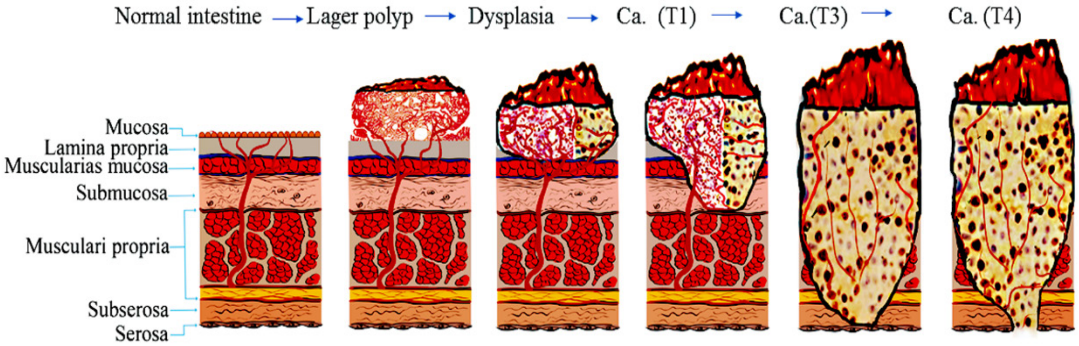


Figure 5: Schematic view of the development of CRC.

The progression of small to lager colorectal adenoma polyps to dysplasia, and then to adenocarcinoma is multi-factorial, with different polyp subtypes patterns in their genetic, immune and microbiome features. Genetic environment, for example adenomas (CAD) are characterized by chromosomal instability (CIN, pre-cancer stage) and show early mutations in the adenomatous polyposis coli (APC) and (KRAS) genes, with dysregulated Wnt, RAS, PI3K, p53 and TGF-β intracellular pathways. Thus, early detection and surgical removal of high-risk lesions from the enlarged polyps or dysplasia of colon (precancer stage) can prevent disease from developing carcinoma stage spreading [52]. STK1p (figure 3B) combined with endoscopy techniques and molecular imaging of the early colonic mucosa (figure 5) are in clinical practice for the screening of adenomatous polyps.

In our meta-analysis we found that the STK1p value increased significantly （$P < 0.0001$ in the following manner: health mucosa (tumor-free) < enlarged polyps < dysplasia < colorectal carcinoma (CRC, T1-3), indicating high sensitivity and specificity of our STK1p assay. It is in agreement with our previous meta-analysis of tumor progression in lung [53], liver [54], breast [55] and gastrointestinal patients (primary data). Those data of four meta-analysis in our present meta-analysis of colorectal tumor together are summarised in figure 6 and 7.

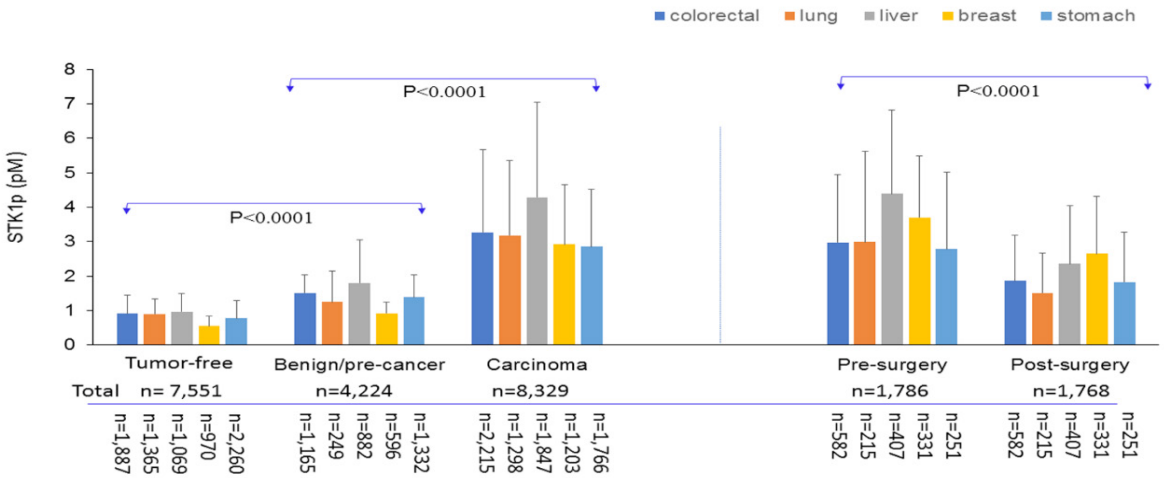


Figure 6: Summary of five meta-analysis in colorectal (present study), lung [53], liver [54], breast [55] and gastrointestinal patients (primary data). *Benign/pre-cancer group (n=4224)；*Carcinoma group （n=8329. *Tumor-free group （n=7551） was used as controls. In all meta-analysis, the STK1p assay was based on TK1-IgY-pAb [10].

As shown in figure 6, the larger data of five meta-analysis indicated that the STK1p assay based on the TK1-IgY-pAb could be used for early detection of benign/pre-cancer patients to prevent them for development into carcinoma. It also demonstrated the need of tumor proliferating biomarker with high sensitivity and specificity, for example STK1p based on the TK1-IgY-pAb developed by us.

Although the original Dukes staging system has been modified several times, the extent of cancer invasion through the bowel wall and that of regional lymph node invasion is still the mainstay of TNM staging for CRC. Monitoring the response to surgery is important. As shown in the summary of figure 6, the STK1p levels (n=1,786) significantly declined ≈ 50-30% one month after post-surgery as compared the pre- surgery in patients at clinical stage I-III ($P < 0.0001$), confirmed our previous studies that STK1p is not only useful to predict prognosis, relapse and survival rate, but also to monitor surgery-treatment in breast [56] and stomach carcinomas [57]. On the contrary, in the patients with distant metastases, the STK1p value increased to 173% at 35 days post-operatively while TK activity did not show significantly difference (57). However, in the case of minimally invasive surgery, the half-life time of STK1p in patients with bladder carcinoma [58] and fibrocystic breast [59] was only one week, the STK1p decreased to normal level. It is strongly recommended that STK1p be combined with suitable imager to evaluate the surgery-treatment effect in patients with CRC individual treatment planning.

Obviously, colon cancer (CC) and rectal cancer (RC) are synonymously called colorectal cancer (CRC). CRC is highly heterogeneous at the genetic and molecular level. The differences exist in molecular carcinogenesis, pathology, surgical topography and procedures, and multimodal treatment, thus the CC is different with RC [60, 61]. The right- and left-sided of CC are significantly different regarding epidemiological, clinical, and histological parameters. Patients with right-sided CC have a worse prognosis [62]. The impact of these findings on screening and therapy remains to be defined.

The following parameters in combination with STK1p should be considered when designing a CRC study: i) CRC is a heterogeneous disease [60], the majority of which is developed from polyp precursors [50, 51]. Therefore, a complete study should use tools useful for the early detection, diagnosis, prognosis and management of CRC development from neoplasms; ii) Since CC and RC are two different types of malignancy[61], they should be evaluated separately; the same goes for right-and left-sided CC and RC [62]; iii) While monitoring the effect of the treatment, the STK1p levels may change depending on clinical stage/grade and tumor type on an individual basis; iv) The STK1p value in patients of three subunits of CRC may be differ among different living areas, depending on the genetic properties and living conditions of the patients individually, studies should include those data in different health centres and oncology hospitals.

The TK1 in serum is stable due to that TK1 in serum formed a native macromolecule complex (730 KD) [63]**.** Incubation at 56°C for 30 min remained about 70% of the enzyme activity, showing the rather stability of TK1 in serum [64]. However it is important to investigate whether the serum sample can be stored for a long time

for assessment of long-time follow-up. To be able to perform such a study it is often needed to work with stored serum samples for shorter or longer times. The collected serum samples can be stored in -80° C, for years before one-time analysis. There is absence of deep freezing at -80° C in some hospitals, serum samples are stored at -20° C for some years. We performed an investigation on serum TK1 concentration (STK1p) to see how the quality is affected after storage at -20° C for 10 years. The serum samples from breast and head & neck malignant patients were collected from Karolinska University Hospital, Sweden and the serum samples of the lung malignant patients were collected at Hubei Tumour Hospital, Wuhan, China. Serum samples were collected in the morning on fasting condition, collected without anticoagulant and then centrifuged at 800×g for 5-8 min. STK1p was determined within 3 hours. The results of the STK1p of patients with breast [16], lung, head & neck carcinomas [12] have been reported. The sera were divided into 4-6 tubes of 200 µl each, at dry-ice and then kept at -20° C and stored for 10 years. At the time for reanalysis after 10 years, 69 serum samples were randomly collected from the cohort of 109 serum samples and re-analysed for STK1p. The hemolysis, lipolysis, precipitation and repeated thawing of serum samples shall not be used. The TK1 in serum were detected by the TK1-IgY-pAb. STK1p value was calculated and expressed as pM. Samples were in duplicate analysis. Results in figure 7 showed that the quality of TK1 in serum is not affected significantly by keeping at -20° C for at least 10 years. This finding opens up for the use of deep-freezing clinical serum samples less than -80° C, at least at -20° C, makes it possible to perform long-time follow-up of tumour patients or determine early tumour risk progress for evaluating the prognosis of patients.

All patients gave informed consent to participate in this investigation, which was conducted in accordance with the Declaration of the 1964 Helsinki *declaration* and the Harmonized Tripartite Guideline for Good Clinical Practice from the International Conference on Harmonization. The collection of the serum samples was performed by permission of the Committee on Research Ethics at Karolinska University Hospital, Sweden (No. 388/01).

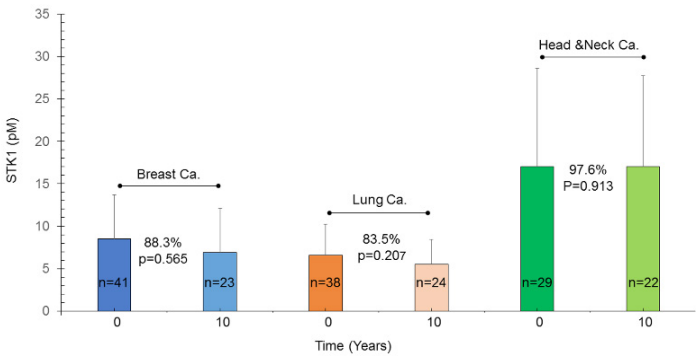


Figure 7: Quality Changes of Deep-Freeze Serum Thymidine Kinase 1 after storage at -20° C during the time of 10 years. Of 69 serum samples were randomly collected from the cohort of 109 serum samples and re-analyzed by STK1p assay. Ca: carcinoma group. Mean values, standard deviation and T-test were determined by SPSS Statistics (V20.0, IBM, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

It shall note that this meta-analysis is a retrospective network meta-analysis and might be replaced by a large randomized clinical trial. A network meta-analysis based on prospective individual patient data meta-analysis are recommended for more reliable assessment of survival or treatment efficacy of patients. This will avoid the influence of bias. We are planning a prospective individual patient data meta-analysis in patients with right-and left-sided CC and RC in relation to STK1p following a nonrandomized individual adapted treatment.

In summary, STK1p assay based on the TK1-IgY-pAb could potentially be used for early detection of polyp/dysplasia to prevent their future development into colorectal carcinoma and give the patient a best chance of cure, as well as for individual clinical kinetic monitoring of the results of surgery in patients with right and left-sided CC and RC. The combination of STK1p with colorectal imaging tools (ex. colonoscopy) after treatment can provide a precise evaluation of the results of the therapy. Together with the use of colorectal-related biomarkers, tumor stage and grade for predicting the risk of relapse, STK1p can help doctors develop more accurate, individualized and rational treatment plans for patients.

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Conflict of Interest Statement: All authors declare no conflict of interest, except for JZ who is the president of SSTK Ltd, the company that produce the STK1p assay used in this study.

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