Clinical Biochemistry 46 (2013) 1453–1461

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Clinical significance of kallikrein-related peptidase (*KLK10*) mRNA expression in colorectal cancer

Dimitra K. Alexopoulou [a], Iordanis N. Papadopoulos [b], Andreas Scorilas [a,*]

[a] Department of Biochemistry and Molecular Biology, University of Athens, Athens GR-15701, Greece
[b] Fourth Surgery Department, Medical School, University General Hospital "Attikon", 1 Rimini Street, Athens GR-12462, Greece

ARTICLE INFO

Article history:
Received 18 December 2012
Received in revised form 22 February 2013
Accepted 3 March 2013
Available online 13 March 2013

Keywords:
Colon cancer
Tissue kallikreins
KLKs
Cancer biomarkers
Real-time PCR

ABSTRACT

Objectives: Colorectal cancer (CRC) is one of the three most common cancers in both genders. Even though several biomarkers are in use in diagnosis and prognosis of the disease, they are marred by limited specificity and sensitivity. The human kallikrein-related peptidase 10 (*KLK10*) gene is a member of the human tissue kallikrein family. Because prostate specific antigen (PSA), the best biomarker for detecting and monitoring prostate cancer, is a member of this family, many other members, including KLK10, have been widely examined as novel biomarkers for different cancer types. In previous studies, KLK10 has been proposed as a diagnostic biomarker for ovarian carcinoma, while its methylation on exon 3 has been proposed as a prognostic marker for early-stage breast cancer patients. The purpose of this study was to analyse *KLK10* mRNA expression and examine its prognostic value and potential clinical application as a novel molecular tissue biomarker in CRC.

Design and methods: The study group consisted of 190 colorectal samples. Total RNA was extracted from pulverised tissues and cDNA was prepared by reverse transcription. *KLK10* was amplified by real-time PCR. *B2M* was used as a reference gene and HT-29 cells as positive control.

Results: *KLK10* expression was significantly higher in cancer tissues ($P < 0.001$). Tumours of advanced TNM and Dukes' stage showed high *KLK10* expression status ($P = 0.036$; $P = 0.025$). Patients with high *KLK10* expression had a shorter disease-free and overall survival rates ($P = 0.014$; $P = 0.020$).

Conclusion: Our results suggest that *KLK10* may serve as a new marker of unfavourable prognosis of colorectal cancer.

Introduction

Colorectal cancer (CRC) is the third most common cancer in both men and women. Incidence rates have been decreasing for the best part of the last two decades. The decline has largely been attributed to increases in the use of CRC screening tests that allow the detection and removal of colorectal polyps before their progress to cancer. In contrast to the overall declining rates, among adults younger than 50 years and in average risk, for which screening is not recommended, CRC incidence rates have been increasing by about 2% per year since 1994, in both men and women. During the same period, mortality rates have declined in both genders. The declining overall incidence and mortality rates reflect improvements in early detection and treatment of CRC [1].

The usual diagnostic procedure is endoscopy and biopsy of the tumour. Current preoperative staging procedures include a full medical history review and physical examination, blood counts, complete biochemistry profile and serum markers, abdominal and pelvic computed tomography (CT) scans, and a plain chest X-ray [2]. The number of the lymph nodes that are surgically removed is used for the staging of CRC and is correlated with patient survival [3].

Several prognostic and predictive markers have been identified, although very few of them are currently used in clinical practice. Microsatellite instability (MSI) is one prognostic factor in use for cancer recurrence and prediction of overall survival in stage II and III patients [4]. Mutations in KRAS are clinically used as a predictor for poor response to treatment with anti-EGFR antibodies in patients with metastatic CRC [5,6]. Other identified prognostic markers, which however are not currently used in clinical practice, include loss of heterozygosity of 18q [7], cancer antigens such as CEA, CA19-9 [8], and RCAS1 [9], enzymes such as thymidylate synthetase [10] and *L*-DOPA decarboxylase [11], apoptosis-related genes, e.g. *BCL2* [12] and *BCL2L12* [13]; however, these molecules are not currently used in clinical practice. No predictive marker for response to adjuvant therapy has yet reached clinical use, although some studies show that MSI tumours are resistant to treatment with fluorouracil [14,15]. CRC is a complex disease and the serum-based tumour markers already in use only play a limited role, due to a lack of specificity and sensitivity. There is an

* Corresponding author at: Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Athens, Panepistimiopolis, 15701 Athens, Greece. Fax: +30 210 727 4158.
E-mail address: ascorilas@biol.uoa.gr (A. Scorilas).

0009-9120/$ – see front matter
http://dx.doi.org/10.1016/j.clinbiochem.2013.03.002

urgent need for identification of new biomarkers if a more effective diagnosis, prognosis and response to treatment is to be achieved.

Human tissue kallikrein-related peptidases (KLKs) constitute the largest contiguous cluster of protease genes with no intervention from other genes. They are located in the chromosomal region 19q13.3–q13.4 and they encode for 15 highly conserved trypsin- or chymotrypsin-like serine proteases [16]. Tissue KLKs were first divided into "classical" and "non-classical" members. The term "classical" refers to the three members that were first identified: KLK1, KLK2 and KLK3 (also known as PSA). The remaining 12 members of the family that were discovered last, namely KLK4-KLK15, are mentioned as "non-classical" [17]. Due to another classification that was adopted later on, only KLK1 still holds the "kallikrein" name, whereas the rest of the KLKs (KLK2-KLK15) are characterised as kallikrein-related peptidases [18]. The KLK family is an attractive field of study because one of its "classical" members, the famous PSA/KLK3, is the most acceptable and broadly used cancer biomarker until today [19]. Moreover, several KLKs have been shown to possess prognostic and/or predictive value in CRC, including *KLK4* [20] and *KLK7* mRNA [21], as well as KLK5, KLK7, and KLK11 proteins [22].

The human kallikrein-related peptidase 10 (*KLK10*) gene was cloned by using subtractive hybridisation between normal mammary epithelium cells strain and its radiation-transformed carcinogenic derivative. It was first named *NES1* (Normal Epithelial cell-Specific 1 gene), because of its selective expression in normal mammary epithelial cells [23]. The *KLK10* gene is about 5.5 kb in length and consists of six exons (one non-coding and five coding exons). Owing to alternative splicing, which is common in all human *KLK*s and many other genes located on the genomic locus 19q13.3–q13.4 [24], the *KLK10* gene is transcribed into three distinct splice variants: splice variant 1 (SV1), splice variant 2 (SV2), and splice variant 3 (SV3). All variants have a different first non-coding exon, whereas all five coding exons are identical, with the exception of a 3-bp 5′-extension in the first coding exon of SV1 [25].

The *KLK10* gene codes for a 30-kDa secreted serine protease of 276 amino acids. The residues that form the catalytic triad give KLK10 a trypsin-like enzymatic activity [26]. *KLK10* gene expression has been detected in many tissues, such as the salivary gland, skin, colon, fallopian tube, prostate, testis and more, and is detected in various biological fluids, such as the milk of lactating women, seminal plasma, amniotic fluid, male and female serum, and CSF [27]. The immunohistochemical expression pattern of KLK10 has been found to be cytoplasmic and not organ-specific [28,29].

Several studies have been performed in order to evaluate the potential role of KLK10 as a cancer biomarker [30]. Due to its higher levels in the tumour tissue and serum of ovarian cancer patients, the KLK10 has been proposed as a useful serological diagnostic and prognostic marker for ovarian cancer, in combination with CA125, for increased diagnostic sensitivity [31–35]. *KLK10* mRNA expression was also found to be upregulated in colorectal and pancreatic cancer by an *in silico* analysis of EST and SAGE libraries [36]. This analysis was confirmed by another study in which elevated levels of *KLK10* mRNA expression detected in tissue samples by semi-quantitative PCR were associated with CRC progression and unfavourable prognosis [37]. Higher levels of the KLK10 protein have also been observed in CRC tissue extracts, in comparison with normal colorectal samples [22].

In contrast, KLK10 expression has been found to be downregulated in breast and prostate cancer cell lines and in breast tumours [38,39], as well as in testicular cancers [40]. Transfection of KLK10 into a highly aggressive KLK10-negative breast cancer cell line resulted in suppression of the carcinogenic phenotype in nude mice [38]. These results suggested that KLK10 may function as a tumour suppressor [41]. However, when KLK10 levels were measured in serum from women bearing malignant or benign breast tumours as well as from healthy controls, no significant difference was observed [42]. This finding was not surprising, since KLK10 is a secreted protein that is expressed in many tissues. Thus, serum levels would count for the total KLK10 excretion from multiple tissues and it seems difficult to detect a difference caused by the tumour. Nevertheless, such a difference was observed in the serum of ovarian cancer patients [32].

Other research groups have tried to analyse the mechanism of KLK10 loss of expression in some tumours. The results suggested that the hypermethylation of the gene is probably the cause of its silencing; neither a deletion nor a rearrangement of the *KLK10* gene accounted for this downregulation of expression. Since the promoter region is defined to be poor in CpG islands and most cancer cell lines are able to support full or partial transcription from the *KLK10* promoter, another *cis*-acting mechanism was considered responsible for KLK10 loss of expression. Finally, the methylation sites were discovered within CpG-rich exons 2–4, especially in exon 3 [43–45].

In the present study, we have analysed the expression of *KLK10* mRNA in CRC specimens using a highly sensitive and accurate quantitative real-time PCR (qRT-PCR) methodology based on the SYBR Green chemistry, and we demonstrated its prognostic potential and clinical utility as a novel tissue biomarker in CRC.

Materials and methods

Colon tissue samples

The study group consisted of 136 patients who underwent surgery for primary CRC at the Fourth Surgery Department, University General Hospital "Attikon", Athens, Greece, between 2000 and 2009. For 54 of them, normal adjacent tissue was also available. All tissue specimens were frozen in liquid nitrogen immediately after surgery, until pulverised and dissolved in TRI Reagent (Ambion (Europe) Ltd., Huntingdon, UK).

Tumour tissues were histologically characterised by a pathologist and a database containing clinicopathological data of patients was built for statistical analysis. Informed consent was obtained from patients participating in the study. The study was approved by the institutional review board of the University General Hospital "Attikon" (Athens, Greece) and conducted in accordance with the ethical standards of the World Medical Association Declaration of Helsinki (version: 2008).

Patient age varied between 35.0 and 93.0 years with a mean ± SE of 66.5 ± 1.1, and tumour size from 1.00 up to 14.00 cm with a mean ± SE of 4.75 ± 0.19. Follow-up information was available for 121 patients and included disease-free survival (DFS) and overall survival (OS) status along with the dates of recurrence and death, as well as cause of death.

Cell line

The colon adenocarcinoma grade II cell line HT-29, used in this study as a calibrator in real-time PCR, was cultured in McCoy's 5A medium (PAA Laboratories GmbH) supplemented with 10% foetal bovine serum (South America Origin), at 37 °C, 5% CO_2, according to ATCC instructions. Cells were grown at a confluence up to 80%, and then harvested with the proteolytic enzyme trypsin. After a short mild centrifugation, the cell pellet was diluted in TRI Reagent (Ambion) for RNA isolation.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from HT-29 cells and colon tissue specimens using TRI Reagent, following the manufacturer's instruction. For the safe storage of RNA at −80 °C until use, total RNA samples were diluted in an RNA Storage Solution (Ambion (Europe) Ltd.). RNA concentration and its purity were determined spectrophotometrically. 2 μg of total RNA was reverse-transcribed into first-strand cDNA using the Superscript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo-dT as primer, following the manufacturer`s instructions. The final reaction volume was 20 μL.