Decreased Expression of KAI1 in Colorectal Cancer Significantly Associate with the Cancer Metastasis

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ABSTRACT

The dramatic increase in the prevalence of colorectal cancer is the most serious health challenges in the world. The higher rate of morbidity for this cancer is positively correlated with metastasis. For reduction in morbidity and alleviation of cancer pain, early detection is inevitable. Recently, it has been shown that KAI1 /CD82 gene has a critical role in the suppression of metastasis. Thus, a change in KAI1 expression is important and could be used as a useful marker for the identification of several cancers such as colorectal, prostate and breast cancer. In this survey, we obtained 52 cancer specimens from different stages of cancer (stages 1, 2, 3 and 4) and ten normal specimens as control from unrelated patients were included in this study. The patients in the study were asked to fill in a questionnaire regarding the concept of consent. Real‑time reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) were performed for evaluating gene expression at RNA and protein level, respectively. The results were analyzed by using a one-way ANOVA followed by post-hoc tests. Real-time RT-PCR quantification and IHC analysis revealed that the expression of KAI1 was significantly increased in the patient compared with the control (P = 0.036). Further comparison of the data showed that the difference was profound between these two groups; stage1 and stage3 with respect to the KAI1 gene expression (P <0.05).

Key words: *KAI1*, Metastasis, Colorectal Cancer

INTRODUCTION

KAI1 orCD82 is known as a tumor suppressor gene and suppressor of the metastasis by inhibiting proliferation and invasion of the cancerous cells [1]. Studies have shown that KAI1 level of expression could be considered among the beneficial markers for evaluating the metastatic tumors function. KAI1 was subject to evaluation in a wide range of tumors during the past decade. The results have shown that KAI1 is expressed in all normal tissues, while the expression could be different in various tissues. The expression of KAI1 in solid tumors could be considered as a good prognosis factor for identification of the advanced stages of cancer (clinically).

In Studies have indicated an inverse relationship between levels of KAI1 expression and progression toward invasion in many types of cancers, such as prostate colon, lung, liver, and thyroid cancers, respectively. Also, there is an inverse relationship between the amount of KAI1

expression and metastasis in lymph nodes or liver. Therefore, detecting the amount of KAI1 expression in each level of cancer is important, especially with regard to metastasis, as the exact expression level for this gene is special in cancers. A decreased expression level of KAI1 in cellular invasion and metastasis is due to a complex genetic mechanism which probably transcription factors are involved, among which, P53, NFKB, and β-catenin [2-4].

On the other hand, cColorectal cancer (CRC) is one of the most prevalent cancers in the world. Many environmental in addition to inheritance factors are causes of the increased prevalence of these cancers. Statistics have shown that cancer is the third cause of fatality after heart disease and accident in Iran.

On the other hand colorectal cancer (CRC) is the fourth common type of cancers in worldwide and the third in Iran. The aim of this investigation is to evaluate the quantitative expression at mRNA level, as well as KAI1 protein expression in CRC patients and its correlation with the pathological, clinical, and demographical aspect of the patients in order to introduce a marker for identifying the tumor levels, especially for metastasis and invasion.

MATERIALS AND METHODS

Patient selection

Fifty-two tumor and ten normal biopsy specimens were snap-frozen in the liquid nitrogen and stored at −80 °C up to use for real time RT-PCR. In addition, 46 formalin fixed, paraffin embedded (FFPE) tissues were included in the Immunohistochemistry (IHC) analysis. The tumorous tissues were obtained from four stages (Stage 1, Stage 2, and Stage 3 plus metastasis). Tumorous and normal specimens were obtained from patients who had undergone diagnosis surgery at Tehran Imam Khomeini hospital. Specimens were divided into four groups, Group 1 included normal specimens, Group 2 included Stage 1 CRC specimens, Group 3 included Stage 2 CRC specimens, and Group 4 included the Stage 3 and metastasis specimens. The analysis was also included the demographic information such as age, sex, the amount of hemoglobin, and smoking habit. The pathological reports and clinical history were taken at the time of surgery. Staging of the tumors was done according to TNM (tumor-node-metastasis). In addition, tumor samples were from patient who had not been the subject of drug therapy (chemotherapy, or radiotherapy). Also they did not have the hereditary polyposis CRC or Crohn's disease. The ethics of the study was approved by the Medical Ethics Committee of the Tumor Bank of Iran. Informed consents were obtained from patients following explanation of the purpose of the study and application of the resected specimens. The classification of the groups was done as follows: group 1 or control, group 2 that was included in the Stage 1, group 3 involved of the samples from stage 2 patients, and the group 4 which was composed of the tissues from patients with metastasis plus Stage 3 of the staging according to TNM protocol. The control group's specimens were obtained from normal tissues.

Tissue processing, RNA extraction, cDNA synthesis

Total RNA was extracted from human CRC and normal tissues, with Easy Blue solution buffer (Intron Biotechnology Co.) according to manufacturer protocol. Two pairs of primer were designed with oligo7 and gene runner software and were blasted applying www.pubmed.com for validating KAI1 forward primer: 5′-TCACCTACCCCTGTTCCTGCGA-3′ as well as the reverse primer: 5′-ACCTTCTCCATGCAGCCCTCCT-3′. The same approach was used for beta-actin forward primer 5′-CTTGATGTCACGGACGATT-3′, and for reverse primer 5′-CACGGCATTGTCACCAACT-3′, respectively. cDNA was synthesized and was used in quantitative real-time polymerase chain reaction for 52 specimens under investigation.

Real time reverse transcription polymerase chain reaction

The cDNA synthesized was used in quantitative real-time polymerase chain reaction for 52 specimens under investigation. Subsequently, as an independent predictor of the patients' outcome, the expression levels of KAI1 gene was investigated for different stages of the CRC. In order to increase the validity of the results, eliminating erroneous amplification, and avoiding possible DNA contamination, the primers were designed for exon–exon junction of the KAI1. Gene expression was assessed in triplicate and each reaction mixture was carried out in 19 µl total volume including 2µl of 10x reaction buffer, 5.0 µl dNTP mix, 2 µl of the MgCl 50mM, 1 µl of the forward and reverse primers, plus 5U enzyme 3.0µl.

The thermal cycling program was included of 4 min initial step at 94 °C, and 35cycles of 45 sec at 94 °C, 45 sec annealing step at 57 °C for KAI1 and 58 °C for internal control gene primer. For normalizing gene expression level, we checked four internal control genes, among which, beta‑actin was chosen as internal control. Cycle threshold (CT) was chosen for comparing raw data obtained from rotor‑gene corbet‑6000 series thermal cycler and used for analysis of gene expression level. For all reactions, the negative control was included in order to determine non-specification amplification. We also used the serial dilution of a positive control specimen for both target and internal control genes and were calculated the slope of the yielded diagram. The expression level of KAI1 was analyzed by $\triangle\triangle$CT method [21]. Furthermore, the PCR products were checked on 2.5% agarose gel containing ethidium bromide applying 1x TBE and photographed.

Protein assay

For KAI1 expression analysis at the protein level, the examination was executed on the FFPE 4μm thick tissues sections. The mounted tissue sections on slides were deparaffinized, rehydrated, and washed in 3% H2O2. Antigen retrieval was done applying the microwave method in 0.1M of citrate buffer and blocking stage was done by incubating tissues with 5% bovine serum albumin (BSA) at 4°C overnight. After removal of BSA, sections were incubated with diluted KAI1 primary antibody (Anti KAI1 antibody [1A3] (ab47153) for 1 h at room temperature. The sections were incubated with diluted (1:200 in 1% of phosphate buffered saline [PBS]) and biotinylated with secondary antibody (Dako Corporation, Carpinteria, CA, USA) for 30 min at room temperature and washed with PBS. Antibodies sitting bonds were visualized with Diaminobenzidine (DAB, Sigma, StLouis, USA) and contrasted with hematoxylin. Finally analyzed obtained picture of an optical microscope with imageJ software and classified the obtained data [5].

Statistical analysis

In this study, we used of SPSS version 20 software (IBM, New York, NY, USA) was used for statistical analysis and the parametric condition was checked with Kolmogorov–Smirnov test. One‑way ANOVA was used for quantitative data test, followed by post hoc Tukey's test to demonstrate the significance of the difference between groups. The qualitative data significance and relations were checked with Chi‑square test. For real‑time

PCR analysis, we used $\triangle$CT and $2^{-\triangle\triangle CT}$ formulas. For the analyzed photos obtained from IHC, we used ImageJ software and classified percentage of software in 0–25% =1+, 25–50% = 2+, 75–100% = 3+.

RESULTS

Evaluation of Quantitative and qualitative of mRNA extraction

Evaluating the quantity of the total RNA extracted showed that all of RNA's had a concentration more than 1000 ng/μL and the 260/280 OD ratio of 1.8–2. The quality of total RNA extracted which were assessed by electrophoresis in a 1.5% agarose gel, was appropriate for doing the Q-PCR [Figure 1, the panel A].

KAI1 and β-actin reverse transcription polymerase chain reaction:

Identify the quality of the designed primers and find the best annealing temperature, we checked cDNA specimen with RT‑PCR and followed the PCR product on the 1.5% agarose gel electrophoresis. The electrophoretic pattern of the several samples are shown in Figure 1; the panel B shows amplified products for beta‑actin and the panel C for KAI1, respectively. Similar results were found for all other examined specimens. The obtained results indicate that KAI1 gene expression subjects to a decreased level of expression in CRC stages.
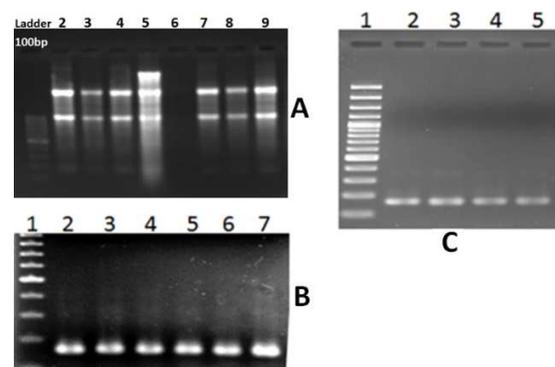

Figure 1: Panel A- 1.5% agarose gel electrophoresis total RNA. Demonstrate extracted mRNA quality Panel B-A pattern of amplified cDNA fragments for some specimens in normal and tumoral stages by β-actin gene primers on 1.5% agarose (161 bp). From left to right; lane1: ladder 100bp, lanes 2-4: some specimens in normal. Lanes 5-7: some specimens in tumoral stages. Panel C- A pattern of amplified cDNA fragments for some specimens in normal and tumoral stages by KAI1 primers on 1.5% agarose (160 bp). From left to right; lane1: ladder 100bp, lanes 2-3: some specimens in normal. Lane 4: a tumoral specimen in stage 1. Lane5: a tumoral specimen in stage 2.

Quantitative analysis of KAI1 gene expression by real‑time polymerase chain reaction:

KAI1 gene expression was significantly decreased totally in CRC stages compared to the normal samples (P = 0.036). To compare KAI1 gene expression in four groups (normal, Stage1, Stage2, Stage3) one way ANOVA test was used. Based on this test, a significant difference in the KAI1 expression among the four groups was observed. (P = 0.036). To identify the significance of the difference between studied groups a pairwise analysis was done for comparison using Post Hoc test. The analysis of the results has indicated a significant difference between Stage1 and Stage3 for KAI1 gene expression (P <0.05).

by other investigators which will mention in the discussion**,** it was expected to observe a decline in KAI1 gene expression from normal tissues toward stage1 of CRC followed by further progress into Stage 2 and Stage3. To test this assumption one way ANOVA test was conducted applying Contrast tests instead of Post Hoc. The analysis resulted in observing a significant difference between the Stage1 and Stage3 in KAI1 gene expression (P = 0.009) and again, the above results obtained. In fact, the relationship between KAI1 gene expression in cancer process and normal tissue was as a linear (Figure 2), which means that expression of this gene with the same slope at different stages of cancer Changes.
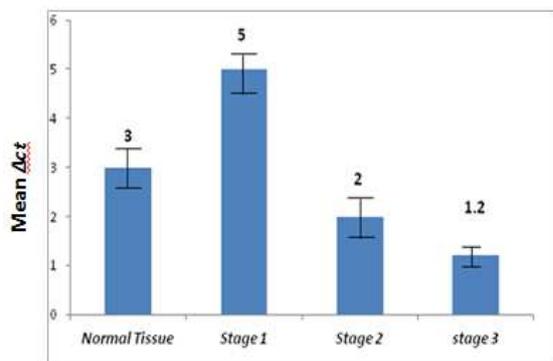

Figure 2: KAI1 gene expression according to mean Δct/groups. The gene expression of KAI1 decreases with advances in cancer stages

The ratio of gene expression was calculated by $2^{-\Delta\Delta ct}$ and was found that KAI1 gene expression was decreased in cancer stages compared to the normal counterparts. It is necessary to notify that the efficiency of PCR in real time for KAI1 and beta actin genes were 2. The amplification plot of KAI1

gene (A), melting curve (B), and the standard curve of KAI1 gene (C) are shown in the Figure 3.
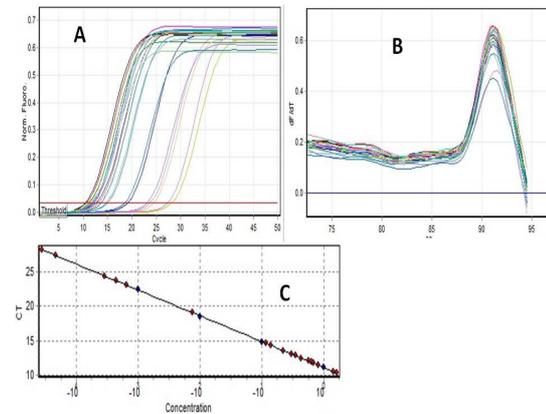

Figure 3: The Q-PCR amplification characteristics of the KAI1. Amplification plot (A) melting curve (B) and the standard curve for KAI1 (C) are shown in this figure
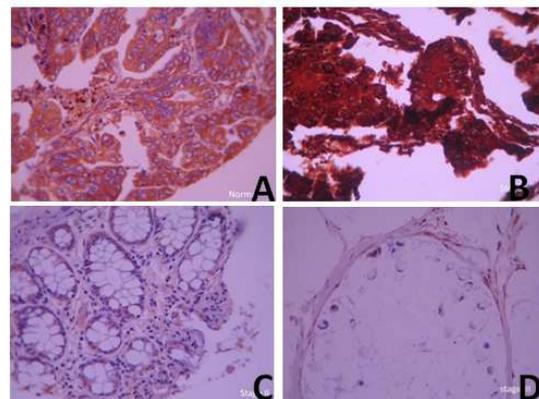

Figure 4: The immunohistochemistry result: photograph obtained immunohistochemistry with ocular microscope, the KAI1 protein expression in (a) normal tissue microarray (magnification X100), (b) Stage I Tumor colorectal cancer (magnification X400), (c) Stage II Tumor colorectal cancer (magnification X100), (d) Stage III Tumor colorectal cancer (magnification X400), the brown color demonstrate DAB sediment, the gene expression decreased in protein level with cancer stage increasing (a up to d)

Immunohistochemistry, expression of KAI1

To scrutinize results obtained by Q-PCR analysis, we further carried out IHC in order to obtain a visual clue for altered expression of KAI1 at the protein level. The processed tissues were analyzed using the optical microscope at a magnification ranging from 100X to 400X magnification further indicated a significant decreased in the expression of the KAI1 at protein level throughout the stages of the CRC compared to the corresponding normal

specimen. As well, an entire loss of protein expression was observed in metastatic this feature is shown in Figure 4. Stage 1: 46% 2 + and 58% 3+, Stage 2: 13% 1+, 83% 2+, 4% 3+, Stage 3: 100% 1+.

The relationship Correlation of experimental data with demographic and pathologic factors
In this study, we evaluated the mRNA and protein levels in 52 unrelated patients with colorectal cancer, while none of them had a family history with any type of cancers. 31 (59 %) of the patients were female and 21 (41%) of them were male with a mean age of 60.8+ 13.9 years. The age of patients was raging from 37 to 84 years. 63% (33 out of 52) of these patients were above 50 years old.

As point of race considering the ethnicity, 63.5% of patients or 33 individuals out of 52 were Azari, while 3.5 % of patients including a total number of 7 patient were Gilaki, and 33.5% (i.e., 12 patients) were from other ethnicities. Geographically, most patients (i.e., 66.5%) were from Northern as well as North-west part of Iran. Also, 5.7 % of patients were the smoker or were using opioids.
The obtained pathological reports based on TNM classification showed the following differentiation pattern: 18 tissue samples were found to be in stage I, 10 in Stage II, and 12 in the third and fourth stage (Stage III & IV), in addition to 12 were normal samples table 1.

Furthermore, we evaluated the association between the levels of mRNA and protein expression for KAI1gene with the clinico-pathological feature at P < 0.05 of significance. The statistical analysis of the results showed not a significant relationship between the mRNA expression levels of the KAI1 and demographic and pathologic factors of the patients with CRC (P>0.05).

However, statistical analysis has shown a significant relationship between KAI1 protein expression level and metastasis to the lymph nodes (P = 0.017) as well as stages of cancer (P = 0.00).

In addition, statistical analysis of the results did not show any significant relationship between the stage of cancer progression and age, gender, and TNM factors (P.0.05).

The statistical analysis showed that the results obtained for QRT-PCR and IHC support each other. Also, it was found a direct correlation between the average Δct and protein expression levels (P < 0.05). In the other words, the higher Δct that exists in the advanced tumor stages, indicates lower level of KAI1 protein expression. As in the steps 3 and 4, as well as metastasis, the proprietary of the KAI1 protein expression reaches to zero.

DISCUSSION

The aim of the present study was to the investigate applicability of the KAI1 in determining cancer staging and early stage recognition of the CRC. To achieve this end, we checked KAI1 expression in the level of protein and mRNA. The expression of KAI1 was analyzed in tumor and the adjacent normal non-malignant tissue (the marginal normal tissue 5 cm away from site of the tumor). We found that KAI1 expression was significantly decreased both at protein and mRNA levels, however, the level of reduction was more striking at mRNA level. This observation might indicate the important role of KAI1 inhibition in CRC metastasis. Metastasis is inhibited by multiple mechanisms such as inhibition of cell motility and invasion, promotion of apoptosis, induction of the senescence in tumor cells, as well as secretion of the external β-catenin [6].

Although the real mechanism and inhibitory effect of KAI1 in the infiltration of cancer metastasis are unknown, IHC results show that KAI1 protein is located in the cytoplasm and cell membrane. According to this finding, it could be suggested that the tumor metastasis inhibitory effect of the KAI1 is executed through its involvement in the regulation of the cell adhesion.

The studies have indicated that in CRC development, the level of KSI1 expression is reduced in the metastasis but the scale of expression is different from other cancers. For example in a study carried out in Germany on patients with kidney cancer [7], through the application of northern blotting it was shown a reduction inKAI1 expression. Moreover, IHC examination carried out on tissues of the patients on cancers of colorectal [8], cervical [9], esophagus [10, 11], prostate and bladder [4], breast [12], and pancreas [13] have indicated that expression of KAI1 is reducing through the process of disease development. These findings

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have provided evidence for a significant relationship between the reduction in this protein level and metastasis.

Supporting the above finding, there are other studies that through evaluation of KAI1 mRNA and protein levels have concluded the vital role and involvement of the reduced KAI1 expression in cancer development in many other tissues. Examples of such studies are studies carried out in cancers of prostate and bladder [4], brain [14], endometrial [15], breast [12], neck and head [16, 17], and lung cancer [18,19], in addition to HCC [20]. All the aforementioned studies have shown the decrease in KAI1 expression metastatic sample, whereas, in non-metastatic samples, the level of expression was slightly higher.

a reduced KAI1 expression in CRC, a contradictory report by [21] has shown an increased expression of KAI1 in tumor tissues as much as is 87 percent higher than the normal tissue. Expression of KAI1 in was found to be correlated with severity of tumors. The Expression of KAI1 at mRNA levels in stages II and III were found to be significantly higher than stage IV four (p < 0.03 and p <0.015, respectively).
a conclusion, it could be hypothesized that the effect of a tumor suppressor gene on metastasis to be dependent on its protein product function. A hypothesis that demand further investigation [22]. In conclusion, it could fairly be suggested that KAI1 plays a role as a metastasis suppressor gene. While the so far studies including our present report indicate, this gene is a metastasis suppressor, while further works in future will better unravel its role and giving a clear picture on the overall function of third tumor suppressor gene in cancer development and metastasis.

CONCLUSION

From results obtained in the research by other projects, it can be said that KAI1 can play role in the development of colorectal cancer (CRC) to higher stages. In other words, it may be possible it acts as a good marker to indicate the infiltration, metastasis, and prognosis of colorectal cancer.

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