Risk Assessment in Hereditary Colorectal Cancer Family by Using APC and MSH2 mRNA Gene Expression and Bayesian Analysis

Tjahjadi Robert Tedjasaputra1,*, Mochammad Hatta2, Muhammad Nasrurn Massi2, Rosdiana Natzi3, Ilhamayaa Patellong4, Marcellus Simadibrata5, Ibrahim Labeda6, Rina Masadah7, Muhammad Luthfi Parewangi8, Prihantono9, Andi Asadul Islam10, Agussalim Bukhari11, Budu12, Rinda Nariswati13

1Department of Internal Medicine, Tarakan General Hospital Jakarta, Jl. Kyai Caringin No.7, Jakarta, Indonesia
2Biology Molecular and Immunology Laboratory, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan KM.10, Makassar, Indonesia
3Department of Biochemistry, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan KM.10, Makassar, Indonesia
4Department of Physiology, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan KM.10, Makassar, Indonesia
5Department of Internal Medicine, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No.6, Jakarta, Indonesia
6Department of Gastroenterology Surgery, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan KM.10, Makassar, Indonesia
7Department of Pathology Anatomy, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan KM.10, Makassar, Indonesia
8Department of Internal Medicine, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan KM.10, Makassar, Indonesia
9Department of Oncology Surgery, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan KM.10, Makassar, Indonesia
10Department of Neurosurgery, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan KM.10, Makassar, Indonesia
11Department of Nutrition, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan KM.10, Makassar, Indonesia
12Department of Ophthalmology, Faculty of Medicine, Universitas Hasanuddin, Jl. Perintis Kemerdekaan KM.10, Makassar, Indonesia
13Department of Statistic, School of Computer Science, Bina Nusantara University, Jl. Mandala No.2, Jakarta, Indonesia

*Corresponding author. E-mail: rtedjasaputra@yahoo.com

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Abstract

BACKGROUND: Some of colorectal cancers (CRCs) are familial, however, heterozygote relatives have approximately 80% lifetime risk of cancer. Risk assessment of CRC’s family could be calculated by direct measurement of mRNA gene expression and Bayesian theorem which is modifying initial background of pedigree risk with additional conditional information. This application has not been reported.

METHODS: The cross-sectional translational sequential studies were performed: (1) adenomatous polyposis coli (APC) and MutS homolog (MSH)2 mRNA quantitative RT PCR gene expressions in tissue and whole blood CRC patients; (2) gene expression was determined in matched controls; and (3) pedigree and Bayesian analysis was calculated in the patient’s family of Proband.

RESULTS: Fourty CRC and 31 control subjects were enrolled. The mean blood APC level control’s group was 13,261±670 fold-change (fc) and blood MSH2 level was 12,219±756 fc. The cut-off points for hereditary APC was 12,195 fc and MSH2 was 11,059 fc. The mean APC blood level in CRC subject was 11,578±2,638 fc and MSH2 blood level was 11,411±2,912 fc. There were significant differences APC and MSH level between tissue and blood level in CRC. Eight of 40 CRC subjects had a history of familial CRC. Four patients and 10 Probands were available for recurrence risk evaluation of pedigree analysis, RNA PCR quantitative and Bayesian calculation.

CONCLUSION: There was determined a cut-off point of hereditary mRNA quantitative expression. The APC and MSH2 levels in CRC subjects were significantly lower than controls. Bayesian analysis allowed for the calculation of relative risk in CRC family members and considered in clinical practice.

KEYWORDS: hereditary CRC, APC gene, MSH gene, Bayesian analysis

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Introduction

Colorectal cancer (CRC) is one of the most common forms of cancer. Most cases are sporadic, but a small proportion are familial. Among which two are common hereditary CRCs, familial adenomatous polyposis (FAP) along with their variants attenuated FAP and Lynch syndrome (LS) or hereditary non-polyposis colon cancer (HNPCC). These disorders are inherited as an autosomal dominant trait, and thus relatives of affected person should undergo periodically colonoscopy examined. Heterozygotes for the most commonly mutated FAP and LS gene have approximately 80% lifetime risk for development of cancer.(1) Some other sporadic cancers likely reflect a predisposition to a specific cancer due to familial variant in one or more genes and the other cancers can also show increased familial incidence without fitting a clear-cut Mendelian pattern.(1,2)

The first-degree relatives of colon cancer patient raise their individual’s risk for the disease approximately two- to three-fold. Having the first-degree relative with colon cancer is sufficient to trigger the initiation of colon cancer screening by colonoscopy at the age of 40 years, which is 10 years earlier than the general population. The increased risk is even more pronounced if two or more relatives have had the disease.(2)

Mendelian principles can be used to detect recurrence risk for family members in single-gene inheritance. However, risk calculation may be less than straightforward if there is reduced penetrance or variability of expression, or is the result of a new mutation. Under these circumstances, Mendelian risk estimates can be modified by applying condition probability to the pedigree. The disorder with incomplete penetrance and late onset of autosomal dominant conditions are a two-condition problem in determining the recurrence risk in family member. Empirical recurrence risks can be used for complex trait disorders, however recent research makes empirical recurrence risk obsolete and should thus be replaced with individualized risk based on genotype and environmental exposure.(3-6)

The inherited family colon cancer syndrome of FAP is autosomal dominant for adenomatous polyposis coli (APC) gene, located in chromosome site 5q21. LS is also autosomal dominant for human MutL homolog (hMLH)1 gene, located in chromosome 3p21, human MutS homolog (hMSH)2 in 2p21-22, hMSH6 in 2p16, human postmeiotic segregation (hPMS)1 in 2q31, hPMS2 in 7p22, and tumor-associated calcium signal transducer (TACSTD)1 in 2p21. MSH2 and MLH1 are the most associated with HNPCC. MLH is more often found in sporadic colon cancer.(7)

Previous genetic research studies have focused on DNA sequence polymorphism as the root of individual differences to disease susceptibility. However, studies of individuals mRNA and protein expression analysis have not been done regularly, and errors are rare because proof reading and repair mechanisms make sure that transcription runs smoothly. The editing of RNA is performed by enzymes which target mRNA post-transcriptionally.(8)

Screening by Amsterdam and Bethesda criteria for hereditary CRC LS is often difficult, especially for small member families and late age at onset.(9-13) To assess the risk in hereditary colon cancer relatives, we would need a family history in pedigree as a basic Mendelian principle, and information on the direct molecular expression of that hereditary gene.(9,14)

The calculation of recurrence risk of CRC in family member requires that the clinician has working knowledge and understanding of basic probability theory. To the best of our knowledge, there is no report of the RNA quantitative hereditary gene measurement of APC and MSH2 in relatives of CRC patients. Bayesian’ theorem enables the modification of initial background ‘prior’ risks with additional ‘conditional’ information, thus assess an overall probability or risk for specific events such as carrier status. (15-17) Therefore, the aim of this study was to determine a cut-off point for RNA quantitative hereditary gene measurements, determine APC and MSH2 levels in CRC patients and controls, and finally to use Bayesian analysis to determine the relative risk of family members of CRC patients developing CRC themselves.

Methods

Study Design

We conducted a cross-sectional translational study on 40 CRC’s subject and 30 control’s subjects in Tarakan general hospital and Siloam Lippo village hospital from May 2018 – December 2019. CRC patients underwent colonoscopy for tissue biopsy sample and whole blood sample to measure APC and MSH2 real-time quantitative mRNA expression.

The non-probability consecutive sample for mean difference Student’s t-test analysis between control and CRC was used. Bivariate analysis of the related variables and the gene expression mRNA levels were conducted by t-test or Mann Whitney for numeric data as opposed to X2 or Fischer Exact test for categorical data.
Subject Enrollment

All patients with CRC who underwent biopsy were eligible. The diagnosis of CRC was based on clinical, endoscopic, and histologic findings. Protocol was approved by Health Research Etic Committee University of Hasanuddin (No 884/H4.8.45.31/PP31-Komite/2018) in October 28, 2018. Informed consent was obtained from all enrolled patients prior to inclusion in this study.

The inclusion criteria were: CRC patients; normal controls matched by age, sex, body mass index; and relatives of the patient if there were hereditary and pedigree findings in tissue RNA and blood RNA. Patients were excluded if they had any of the following conditions: history of other cancer, a history of chemotherapy or radiotherapy, inflammatory bowel disease, or refusing to participate in the research study.

Three sequential studies were performed. First, we aimed to discover clinical data: phenotype of colon cancer by colonoscopy, and APC RNA and MSH2 RNA gene expression in tissue and whole blood of CRC patients. The second study aimed to discover RNA gene expression in normal controls by matching. The third study aimed to determine APC and MSH2 expression of blood sample in the family of the patient if the data showed hereditary gene expression; subsequent Bayesian analysis was calculated.

Collecting Samples and Measurement of APC and MSH2 Expression

Tissue samples were taken by colonoscopy biopsy in CRC patients. Whole blood samples of 0.3 mL were taken by 1 cc needle. Both samples were placed in an L6 buffer preservative sample tube separately. We created homemade L6 buffer preservative according to the standard procedures of the RNA extraction Boom method in the Biomolecular Laboratory of Universitas Hasanuddin. For APC and MSH2 mRNA quantitative measurement, we used real time PCR assay. PCR results were analyzed using Bio-Rad CFX Manager 3.1 software (Biorad Laboratories, Hercules, CA, USA).(18-20)

PCR Technique

We used specific oligonucleotide primer gen targets of MSH2 and APC. Beta actin and catenin Beta (CTNNB1) were used as housekeeping gene (internal control). The primers sequences of mRNA MSH2 gene and Beta actin (housekeeping gene) were as follows: MSH2_For: CATCCAGCCCATGCTTGTTGA and MSH2_rev: GCAGTCCACAATGGACACTTC. The primers of Housekeeping gene were as follow: APC for: 5’ TCTTGAAATGAACCCCATAGGAA 3’ and Rev: 5’ TCTTGAAATGAACCCCATAGGAA 3’. The primers sequences of housekeeping gene were as follows: GAPDH for: 5’ CGCTCTCTGGTCTCTCGCT 3’ and GAPDH Rev: 5’ CCATGGTGCTGAGGCCATGT 3’. The Amplicon were APC: 89 bp, GAPDH 81 bp, MSH2 215 bp, Beta actin 109 bp.

Detection of mRNA MSH2 and APC gene expression was performed using specific primer forward and Reverse protocol PCR: by multiplying DNA of the cycle of 94°C in 3 minutes. The cycles were repeated 38 times in 54°C for 30 seconds.(18-20) The primer was designed by Macrogen (Seoul, Korea). The qRT-PCR measurement was performed using Green QRT-PCR master mix kit, one stage. The kit was Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The protocol was optimized for CFX Connect system instrument, real time PCR 96 well 0.1 mL (Biorad Laboratories). The protocol was adjustable by changing dye dilution based on the manufacturer’s recommendations for RT-PCR cycle programs.(21,22)

Bayesian Theorem

The family risk estimates were based on genetic principles, Mendelian carrier history, and direct molecular genetic method for direct detection of the mutation. The probability calculation was performed using Bayesian equation (Prior Probability and Posterior Probability). (2,16)

Recall that (A|B) = (P(B|A) × P(A)) / (P(B)), with prior E(β) = ∫ β p(β|γ,ϑ) dβ, and the posterior probability can be concocted as:

\[
p(β|y, n) = \frac{p(y|β, ϑ) p(β|ϑ)}{\int p(y|u, ϑ) p(β|ϑ) du} = \frac{f(y|β, ϑ)σ(β|ϑ)}{\int f(y|u, ϑ)σ(u|ϑ) du}
\]

Results

A total of 40 patients with CRC and 31 control subjects were enrolled. There was no significant difference in baseline characteristic between CRC patients and controls. The basic characteristic of subjects was showed in Table 1. Eight CRC subjects had a history of family CRC, four of which gave informed consent for evaluation for pedigree of CRC family history. From these 4 CRC family pedigrees, we established 10 Proband which could be evaluated using
Table 1. Baseline characteristic.

| Variable                  | CRC Group (n=40) | Control Group (n=31) | p-value |
|---------------------------|------------------|----------------------|---------|
| Age (years old), Mean±SD  | 56.80±8.40       | 51.61±13.44          | >0.05   |
| Sex, n (%)                |                  |                      |         |
| Male                      | 21 (52.5)        | 13 (41.9)            | >0.05   |
| Female                    | 19 (47.5)        | 18 (58.1)            | >0.05   |
| Body mass index, Mean±SD  | 22.41±3.29       | 23.62±3.41           | >0.05   |

There were no significant differences in basic characteristics between CRC and controls subject.

The obtained histopathological result of 39 CRC subject were adenocarcinoma and one CRC subject was neuroendocrine carcinoma. There were 3 adenocarcinoma subjects with Signet ring cell/mucinous CRC. From 39 CRC subjects, the histopathological differentiation of 26 (66.7%) subjects were well differentiated, 6 (15.4%) subjects were fair differentiated, and 7 (17.9%) subjects were poor differentiated. There was no immunohistochemical (IHC) test nor DNA sequencing mutation test was done.

Table 2 showed the mRNA APC and MSH2 blood expression level from the control group. There was no significant difference between the 1st, 3rd, and 5th percentile. The 5th percentile cut-off point for hereditary APC was 12,195 and MSH was 11,059 fold-change (fc). Meanwhile, Table 3 showed the differences APC and MSH level between tissue and blood level in CRC.

There was significant difference blood APC level between CRC and control subject. There was lower mean value of MSH2 in CRC but not significant difference between CRC and control subject because of the outlier. (Table 4).

Table 5 showed the APC and MSH2 hereditary prevalence in CRC patients. We assumed a hereditary mutation if the RNA APC PCR and RNA MSH2 PCR levels from the blood and tissue of CRC patients less than the cut-off point established in controls.

Of the 40 CRC subjects, there were 20 (50%) subjects who were hereditary subjects based on APC blood test, 13 (32.5%) subjects who were hereditary subjects based on MSH blood level, and 21 (52.5%) subjects based on both APC and MSH level.

The low RNA expression because of incomplete DNA mutation transcription. It will influence the production of amino-acid that represented by three-nucleotide sequence of codon along the mRNA molecule and the protein production. This will impact the function of antigrowth factor of gene in cell cycle.

There were eight CRC subjects who had a history of familial CRC. All of these subjects were in accordance with the mRNA hereditary cut-off point. Four subjects gave permission for us to evaluate their family members for pedigree analysis (Figure 1A-1D). Ten Probands were available to evaluate carrier states based on family history of Mendelian principles and molecular genetic methods for direct detection of the mutation in the affected proband, using Bayesian calculation.

Table 6 showed that APC and MSH2 expression over the family pedigree and Bayesian analysis of Proband. Risk calculation in initial background ‘prior’ risks of hereditary pedigree to be modified by ‘conditional’ information of age and genome analysis (APC and MSH2 to give an overall probability or risk for carrier status in Column ‘phi’).

**Discussion**

The first of several steps in multiplication cells of DNA is copied as transcription into RNA especially mRNA by RNA polymerase. This is the base of gene expression which can be

| Variable                  | Value (n=40)               |
|---------------------------|----------------------------|
| APC blood, Median (Range) | 12,156.50 (5,848-15,035)   |
| APC tissue, Mean±SD       | 8,147.77±1,875.12           |
| MSH blood, Median (Range) | 11,411.05 (4,230-14,559)    |
| MSH tissue, Median (Range)| 7,485 (4,174-14,218)        |
Table 4. Blood APC and MSH2 gene expression comparison.

| Gene Expression (Blood) | CRC Group (n=40) | Control Group (n=31) | p-value |
|-------------------------|------------------|----------------------|---------|
| APC                     |                  |                      |         |
| Median (Range)          | 12,156.50 (5,848.00–15,035.00) fc | 13,260.00 (12,080–14,376.00) fc | 0.014 |
| Mean±SD                 | 11,578.68±2,638.23 fc | 13,261.74±670.56 Fc | 0.014 |
| MSH2                    |                  |                      |         |
| Median (Range)          | 12,554.50 (4,230.00–14,559.00) fc | 12,146.00 (11,029.00–13,633.00) fc | 0.116 |
| Mean±SD                 | 11,411.05±2,912.45 fc | 12,219.87±756.87 Fc | 0.465 |

Mann-Whitney test of blood APC shows p=0.014 (significant difference blood level APC between CRC subject and control subject). Mann-Whitney MSH blood test p=0.116 (not significant difference between CRC subject and control. fc: fold-change.

The majority of people with cancer-positive family members are not likely to have a cancer-predisposing syndrome. However, the level of risk for persons with a family history of bowel cancer depends on the number of persons with cancer in the family, how closely related they are to the affected relative, and also the age of onset of the affected family member(s).

The APC and MSH2 expression of CRC patients showed different levels of either APC or MSH2 between blood and tumor tissue. In our 40 CRC patients, we found substantial hereditary mutations. Based on laboratory results, the mutation or hereditary prevalence was 50% in subjects based on APC RNA blood test, 32.5% of patients were hereditary subjects based on MSH2 RNA levels, and 52.5% of patients were hereditary subjects based on both APC and MSH levels. The remaining patients are most likely sporadic CRC cases.

The simplest presentation and interpretation of family health history is using a visual family pedigree. A three-generation pedigree is a construct including the health status of first, second, and third-degree relatives within three generations of the family of the patient. This format clearly detected in the tissue or blood. In turn, serves as a template for the protein's synthesis through translation. Cancer is caused by DNA mutations that turn on oncogenes or turn off tumor suppressor genes. This leads to cells growing out of control. Two of the most prevalence of hereditary colon cancer are FAP, which is caused by inherited changes in the APC tumor suppressor gene, and LS (hereditary HNPCC) which is caused by mutation in one of the DNA repair genes like MLH1, MSH2, MSH6, PMS2, and epithelial cell adhesion molecule (EPCAM). The mutation of DNA will express the transcription of mRNA either in tumor cells or the cell of whole body include the blood cell.

Many autosomal dominant conditions characteristically present at late age onset, beyond reproductive age. Direct genomic detection of mutation in a patient’s or family members for a molecular diagnosis has now become standard of care for many conditions.(2) DNA or RNA sample analyses are available from accessible tissues, buccal scraping, blood sampling, and more invasive testing such as amniocentesis.(2) This is the first report in the RNA quantitative hereditary gene measurement of APC and MSH2 in relatives of CRC patients.

Our findings showed that the means of APC mRNA expression and MSH2 mRNA expression level were not significantly different in terms of low cut-off point between the 1st, 3rd, and 5th percentiles. Therefore, we established the 5th percentile as the cut-off point for APC: 12,195 fc and MSH: 11,059 fc. As far as we know, no previous studies have examined APC and MSH2 expression to determine a similar cut-off point in CRC patients.

Table 5. Hereditary prevalence in CRC subject.

| Gene       | Cut-Off (fc) | Hereditary n (%) | Non-Hereditary n (%) |
|------------|--------------|------------------|----------------------|
| APC        | 12 195.80    | 20 (50.0)        | 20 (50.0)            |
| MSH2       | 11 059.60    | 13 (32.5)        | 27 (67.5)            |
| APC & MSH2 | 21 (52.5)    | 19 (47.5)        |                      |
Figure 1. Pedigree analysis in the hereditary CRC patient. A: Mr. A (67-year-old) family; B: Mrs. A1 (44-year-old) family; C: Mrs. E (62-year-old) family; D: Mrs. A2 (47-year-old) family.
Table 6. Bayesian analysis of Proband.

| Proband | Prior Heredity Pedigree | Age (years old) | APC (fold change) | MSH2 (fold change) | Y | phi | yhat |
|----------|-------------------------|-----------------|-------------------|-------------------|---|-----|------|
| Proband 1 | 0.5                     | 30              | 7290              | 9753              | 1 | 1   | 151.4142 |
| Proband 2 | 0                       | 74              | 13832             | 14209             | 0 | 0.932104 | 2.619474 |
| Proband 3 | 0.5                     | 49              | 8727              | 9567              | 1 | 0.50415 | 0.0166 |
| Proband 4 | 0.5                     | 23              | 9757              | 10320             | 1 | 0.50516 | 0.020641 |
| Proband 5 | 0.5                     | 20              | 14524             | 13073             | 0 | 0.500006 | 2.23E-05 |
| Proband 6 | 0.5                     | 43              | 11676             | 10673             | 1 | 0.5   | 0    |
| Proband 7 | 0                       | 64              | 14020             | 13653             | 0 | 0.5   | 0    |
| Proband 8 | 0.5                     | 52              | 6884              | 7073              | 1 | 0.5   | 0    |
| Proband 9 | 0.5                     | 45              | 14341             | 13295             | 0 | 0.5   | 0    |
| Proband 10 | 0.5                    | 28              | 14609             | 13426             | 0 | 0.5   | 0    |

Cut off-point: $APC=12,195; MSH2=11,059; age: 50 years old.

outlines familial relationships which can help recognize inheritance patterns. This format allows the information to also be easily interpreted by others.(16,17)

Our findings regarding hereditary analysis of Probands in patient family pedigree, based on $APC$ and $MSH2$ RNA PCR quantitative gene expression in the cut-off points of $APC$ 12,195 fc and $MSH2$ 11,059 fc showed high family risk in Probands 1, 3, 4, 6 and 8. The risk calculation could be measured by Bayesian method.

For autosomal dominant inheritance-involved disorders, the factors of reduced penetrance and age of onset must also be considered.(2,3,14,23) Estimation of recurrence risk in family members usually requires careful consideration and must take into account: (1) the diagnosis, its mode of heritance, and epidemiological data relating to the natural history (age of onset); (2) analysis of the family pedigree; and (3) the result of tests, which may include linkage studies using DNA markers or negative mutation analysis, and clinical data from standard investigations. Personalized medicine trying to promote in knowledge about genetic factors and biological mechanisms of disease coupled with unique considerations of an individual's patient care needs to make health care safer and more effective.(24)

Our four CRC patients were evaluated for recurrence risk of family colon cancer by Bayesian analysis (pedigree history, age, $APC$ and $MSH2$ RNA PCR analysis). Patient A, a 67 year-old male (Figure 1A), had delayed onset of hereditary CRC. He has a small family with only 1 son (30 year-old, Proband 1). From the history, pedigree, age, and RNA PCR analysis, his son has almost a 100% hereditary risk of CRC. Patient A1, a 44-year-old female (Figure 1B) who developed CRC at a young age (44 year-old). She had a family history of a father who died from CRC. However, her mother (74 year-old, Proband 2), 93% unlikely has hereditary risk of CRC. Her sister, 49 year-old (Proband 3) has a 50.41% hereditary risk of CRC. Her 23-year-old son (2nd child, Proband 4) has a 50.51% risk of hereditary CRC. Her other son (3rd child, Proband 5) is 20 year-old and is 50% unlikely to have hereditary CRC because of a high level of $APC$ and $MSH$ RNA PCR. Further case is Patient E, a 62 year-old female (Figure 1C) with a big family, but only 1 daughter (Proband 6) could be evaluated. Her father died of an unknown cause but her aunt died of CRC. Proband 6, 43 year-old has a 50% risk of hereditary CRC. Last case is patient E2, a 47 year-old female (Figure 1D). She developed CRC at a young age and has a family history of a father dying of prostate cancer. Her mother (Proband 7) is 64 year-old and has 50% unlikely risk of hereditary CRC. Her older sister (Proband 8), 52 year-old has 50% recurrence risk of hereditary CRC. Her younger sister, (Proband 9), is 45 year-old and is 50% unlikely for hereditary risk of CRC. Her daughter (the 1st child, Proband 10) is 28 year-old and is 50% unlikely to have hereditary risk of CRC. Furthermore, the analysis of recurrence risk of Hereditary Colorectal Cancer ware calculated in 10 probands using family history of pedigree as Mendelian principles and direct detection of $APC$ and $MSH2$ quantitative real time PCR expression.

The final Bayesian analysis of Proband probability (with cut-off point of $APC$: 12,195, cut-off point of $MSH2$: 11,059, and cut-off point of age: 50 years old) showed that Proband 1 100% has a heredity risk of CRC, Proband 2 93% unlikely to have hereditary risk of CRC, Proband 3
50% has a hereditary risk of CRC, Proband 4 50% has a risk of hereditary CRC, Proband 5 50% unlikely to have hereditary CRC, Proband 6 50% has a risk of hereditary, Proband 7 50% unlikely to have hereditary CRC, Proband 8 50% has a risk of hereditary CRC, Proband 9, 50% unlikely to have a hereditary CRC. Proband 10 50% unlikely to have hereditary CRC.

There are two main limitations which should be addressed. Firstly, not all hereditary CRC patients and their families allowed us to evaluate their pedigree history. However, we were able to comprehensively determine the pedigree history of 10 probands and individually calculate the hereditary risk. Secondly, not all hereditary genes were included in the Bayesian Theorem calculations. Future studies should consider expand this analysis with a larger cohort and including with other related genes for Bayesian Theorem calculation. Finally, pedigree history should be considered part of the clinical strategy for prevention of CRC in a patient’s family.

### Conclusion

There was analysis to determine a cut-off point for hereditary mRNA quantitative expression based on APC and MSH2 gene and found significantly lower expression of these genes in CRC subjects compared to control. Bayesian Theorem enables the calculation of the probability of Probands carrier status.

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