MTDH genetic variants in colorectal cancer patients

Sebastian Gnosa1,*, Ivana Ticha1,2,*, Staffan Haapaniemi3 & Xiao-Feng Sun1

The colorectal carcinogenesis is a complex process encompassing genetic alterations. The oncoprotein AEG-1, encoded by the MTDH gene, was shown previously to be involved in colorectal cancer (CRC). The aim of this study was to determine the frequency and the spectrum of MTDH variants in tumor tissue, and their relationship to clinicopathological variables in CRC patients. The study included tumors from 356 unselected CRC patients. Mutation analysis of the MTDH gene, including coding region and adjacent intronic sequences, was performed by direct DNA sequencing. The corresponding normal colorectal tissue was analyzed in the carriers of exonic variant to confirm germline or somatic origin. We detected 42 intronic variants, where 25 were novel. Furthermore, we found 8 exonic variants of which four, one missense (c.977C > G-germline) and three frameshift mutations (c.533delA-somatic, c.1340dupA-unknown origin, c.1731delA-unknown origin), were novel. In silico prediction analyses suggested four deleterious variants (c.232G > T, c.533delA, c.1340dupA, and c.1731delA). There were no correlations between the MTDH variants and tumor stage, differentiation or patient survival.

We described several novel exonic and intronic variants of the MTDH gene. The detection of likely pathogenic truncating mutations and alterations in functional protein domains indicate their clinical significance, although none of the variants had prognostic potential.

Colorectal cancer (CRC) is the third most common cancer in men and the second in women with 1.36 million incidences per year worldwide. About 700,000 estimated deaths per year caused by CRC making it the fourth most common cause of cancer death, accounting for about 8.5% worldwide1. Around 75% of the CRC incidences are sporadic, and the rest of the cases are hereditary or familial CRC, associated with inherited genetic aberrations2. As first proposed by Fearon and Vogelstein in 1990, colorectal carcinogenesis is a complex process implicating accumulation of genetic alterations in oncogenes and tumor suppressor genes3. Several oncogenic aberrations including point mutations, insertions, deletions and gene amplification in KRAS, NRAS, BRAF, MYC, WNT and PIK3CA have been linked to colorectal carcinogenesis and are therefore promising genetic markers for early cancer detection, treatment selection and prognosis3–5.

Current research is devoted to search for new prognostic and predictive biomarkers. The Metadherin gene (MTDH; MIM#610323) encodes for the lysine-rich oncoprotein Astrocyte elevated gene 1 (AEG-1), also called LYRIC, which is highly basic 582 amino acid protein with a molecular mass of 64 kDa6,7. The gene is located at chromosome 8q22 and comprises 12 (coding) exons and spans around 95 kb (PMID: 14980505)8. Amplification of genomic loci 8q22 has been correlated to increased AEG-1 expression9–13. Several functional regions in the AEG-1 protein have been discovered. The AEG-1 protein contains an N-terminal transmembrane domain (amino acid (aa)51–72), three putative nuclear localization signals (aa79–91, aa432–451 and aa561–580) and several protein interaction sites14.

We and others have shown that the AEG-1 mRNA and protein are overexpressed in CRC and other types of cancer compared with the corresponding non-tumor tissue15–19. The AEG-1 protein has been found to be involved in cell proliferation, survival, migration, invasion, apoptosis, angiogenesis, metastasis and treatment resistance when interacting with a variety of proteins and protein complexes14,15,17,20–23. Two studies conducted on blood samples from breast and ovarian cancer patients have analyzed the coding sequence of MTDH, and identified a correlation between the polymorphisms c.1353G > A (rs2331652, p.K451K), and c.1679–6 T > C.
corresponding normal mucosa, which corresponded with their germline origin. (rs150495888), and c.1147G
(rs34735761) and c.1353G (p.K451K), and showed a significant correlation to each other (p < 0.05). Each variant of both clusters was also detected in the corresponding normal mucosa, which corresponded with their germline origin.

Table 1. Exonic variants detected in the MTDH gene in colorectal cancer patients. 4GenBank reference sequence NM_178812 (7667bp mRNA); +1 corresponds to the A of the ATG translation initiation codon.

| Exon | cDNA variant | n (%) | Reference | Predicted mutation effect | in silico prediction | Origin |
|------|--------------|-------|-----------|--------------------------|---------------------|--------|
| 1    | c.160G>A     | 4 (1.1) | rs140652237 | p.V54M polymorphism      | germline            |
| 1    | c.232G>T     | 35 (10) | rs17854373  | p.A78S pathogenic         | germline            |
| 3    | c.533delA    | 1 (0.3) | novel      | p.N178Tfs34 pathogenic    | somatic             |
| 6    | c.949A>G     | 56 (16) | rs17854374  | p.T317A polymorphism      | germline            |
| 6    | c.977C>G     | 1 (0.3) | novel      | p.T326S polymorphism      | germline            |
| 9    | c.1340dupA   | 1 (0.3) | novel      | p.K448Efs67 pathogenic    | N/A                 |
| 9    | c.1353G>A    | 9 (2.5) | rs2331652   | p.K451K polymorphism      | germline            |
| 12   | c.1731delA   | 1 (0.3) | novel      | p.A578Pfs29 pathogenic    | N/A                 |

(rs171026063), and breast cancer susceptibility as well as between the polymorphism −470 G>A and ovarian cancer susceptibility20–24.

However, it is unknown whether mutations in the MTDH gene contribute to tumor progression and have prognostic potential for CRC. The aim of this study was to determine the frequency and the spectrum of MTDH variants in tumor tissue and their relationship to clinicopathological variables (patient gender, age at diagnosis, tumor location, tumor stage, grade of differentiation, recurrence and survival) of CRC patients. To our knowledge, this is the first study analyzing mutations of MTDH in tumor tissue.

Results

Frequency of MTDH variants in CRC patients and cell lines. By direct DNA sequencing of the complete coding sequence of the MTDH gene, we found 50 single nucleotide variants in 356 CRC patient samples (Supplementary Table 1). Eight of the variants were exonic and 42 were in a non-coding region adjacent to an exon. Among them, there were four novel exonic variants (Table 1, Fig. 1) [c.533delA (p.N178Tfs34), c.977C>G (p.T326S), c.1340dupA (p.K448Efs67) and c.1731delA (p.A578Pfs29)], and 25 novel variants in a non-coding region adjacent to exons. All variants found were heterozygous, except for the seven variants c.232G>T, c.382-50C>T, c.568+213delT, c.949A>G, c.1048+131T>G, c.1049-97delA and c.1147+28delE. The genotypic frequency is stated in Supplementary Table 1. There was no MTDH variant in the colon cancer cell lines SW480, SW620 and HCT116 (data not shown).

Several variants co-occurred and two clusters were identified (Supplementary Table 2 and 3). The first cluster of variants with a high linkage included the variants c.160G>A (rs140652237, p.V54M), c.568+213delE (rs43735761) and c.1353G>A (rs2331652, p.K451K), and showed a significant correlation to each other (p < 0.05). The second cluster of variants with a high linkage included c.232G>T (rs17854373, p.A78S), c.382-50C>T (rs16896067), c.949A>G (rs17854374, p.T317A), c.1048+131T>G (rs12675731), c.1049-97delA (rs150495888), and c.1147+28delE (rs76537339; p < 0.05). Each variant of both clusters was also detected in the corresponding normal mucosa, which corresponded with their germline origin.

Intronic MTDH variants in relation to clinicopathological variables. The intronic variants c.382-50C>T (rs16896067), c.1048+131T>G (rs12675731) and c.1353G>A (rs2331652, p.K451K) were more frequent in the patients <72 years old compared to the age group ≥72 years old (p = 0.019, p = 0.047 and p = 0.021, respectively; Supplementary Table 4). The variant c.1048+82delA (rs149869061) was only detected in tumors located in the colon but not those located in the rectum (p = 0.013). We did not find any relationship between the variants and the gender, tumor stage, grade of differentiation, recurrence and patient survival (p > 0.05).

Exonic variants in relation to clinicopathological variables and location in functional protein domains. The 50 exonic variants detected in this study were not missense [c.160G>A (rs140652237, p.V54M), c.232G>T (rs17854373, p.A78S), c.949A>G (rs17854374, p.T317A) and c.977C>G (p.T326S)], one silent [c.1353G>A (rs2331652, p.K451K)], and three frame shift mutations [c.533delA (p.N178Tfs34), c.1340dupA (p.K448Efs67), and c.1731delA (p.A578Pfs29)]. To evaluate whether the exonic variants occurred during colorectal carcinogenesis or whether they are inherited, we analyzed the corresponding normal mucosa of the colon and rectum from the same patients. Frame-shift mutation c.533delA was not detected in the corresponding normal mucosa, and therefore considered as a somatic mutation. The corresponding normal mucosa of the other two frameshift variants was not available, therefore we were not able to assess the somatic or germline status. The other exonic variants were detected also in the corresponding normal mucosa (Table 1). The variant c.232G>T (rs17854373, p.A78S) was more frequent in the patients <72 years old compared to the age group ≥72 years old (p = 0.001; Supplementary Table 4). To evaluate the predicted effects of exonic variants on protein function, six in silico prediction tools were used. The in silico prediction analyses revealed that four of these variants c.232G>T (rs17854373, p.A78S), c.533delA, c.1340dupA and c.1731delA, were deleterious (Table 1, Fig. 1, Supplementary Table 5). The variants, c.533delA, and c.1340dupA, lead to a truncation of the protein while...
the variant, c.1731delA, is predicted to lead to protein prolongation. All three variants were heterozygotic and detected in stage I or II colon cancer with moderate or poor differentiation (Table 2).

We discovered two variants which are located in at least one functional region of the AEG-1 protein. The variant c.160G>A (rs140652237, p.V54M), is located in the transmembrane domain and in the CBP and PLZF binding region. The variant, c.232G>T (rs17854373, p.A78S) is located one amino acid before the N-terminal nuclear localization signal and in the YY1, BCCIP and PLZF binding region. The missense variants, c.949A>G (rs17854374, p.T317A) and c.977C>G (p.T326S), are in an area without known protein interaction.

**Discussion**

Overexpression of the oncogene AEG-1 has been reported in several types of cancers and was correlated to increased cell proliferation, invasion, survival and treatment resistance11,13,17,20–23. Numerous studies have shown that overexpression of AEG-1 is due to amplification of the genomic loci at chromosome 8q22, activation of up-stream signaling as well as deregulation of several miRNAs8–13,25–32. However, it remains largely unclear whether mutations in the MTDH gene contribute to its oncogenic properties. In the present study, we therefore examined the frequency and spectrum of MTDH variants, and their relationship to clinicopathological variables.
in 356 CRC patients including tumor tissue as well as in three colon cancer cell lines. In total, we detected 42
intronic variants, 26 of which were novel. Furthermore, we found eight exonic variants of which four variants,
one missense (c.977C > G) and three frameshift mutations (c.533delA, c.1731delA, c.1340dupA), were novel. The
three frameshift variants are likely pathogenic.

Correlation analyses between recurrent variants and clinicopathological variables revealed that the intronic
variant, c.1048 + 82 delA (rs149869061), was only detected in tumors located in the colon but not those located
in the rectum. In a previous study, we found significantly lower expression of the AEG-1 mRNA in the colon
compared to the rectum16. Whether the intronic variant has an influence on the mRNA expression or stability
needs further investigation.

The variants, c.232G > T (rs17854373, p.A78S), c.382–50C > T (rs16896067), c.1048 + 131T > G (rs12675731) and
c.1353G > A (rs2331652) were both frequently detected in blood samples from breast cancer patients (52% and 22%,
respectively) and from healthy controls (36% and 11%, respectively), and both variants have been correlated to breast cancer susceptibility in a Chinese study24. Compared to their results, in the present study the variants, c.1353G > A (rs2331652) and c.1679–6T > C
(rs17026063), were very rare (2.5% and 0.3%, respectively). The different frequencies in the two studies could be
due to the divergence between the ethnic groups (Chinese versus Caucasian), DNA origins and disease mech-

Table 2. Exonic MTDH variants in relation to clinicopathological variables of colorectal cancer patients.

| Characteristics | c.160G > A p.V54M rs140652237 | c.232G > T p.A78S rs17854373 het/hom | c.533delA p.N1873TIs34 novel | c.949A > G p.T317A rs17854374 het/hom | c.977C > G p.T326S novel | c.1340dupA p.K448Ef75 novel | c.1353G > A p.K451K rs2331652 | c.1731delA p.A578Pfx32 novel |
|----------------|-------------------------------|-----------------------------------|-------------------------------|---------------------------|------------------------|----------------------------|--------------------------------|------------------------------|
| Gender         |                               |                                   |                               |                           |                        |                           |                               |                              |
| Male           | 2                             | 18/2                              | 1                             | 28/2                      | 0                      | 1                          | 4                             | 0                            |
| Female         | 2                             | 14/1                              | 0                             | 25/1                      | 1                      | 0                          | 5                             | 1                            |
| Age at diagnosis (mean) |                       |                                   |                               |                           |                        |                           |                               |                              |
| <72 years      | 3                             | 21/2                              | 0                             | 27/2                      | 1                      | 0                          | 7                             | 1                            |
| ≥72 years      | 1                             | 11/1                              | 1                             | 26/1                      | 0                      | 1                          | 2                             | 0                            |
| Tumor location |                               |                                   |                               |                           |                        |                           |                               |                              |
| Colon          | 2                             | 20/2                              | 1                             | 29/2                      | 0                      | 1                          | 5                             | 1                            |
| Rectum         | 2                             | 12/1                              | 0                             | 24/1                      | 1                      | 0                          | 4                             | 0                            |
| Tumor stage    |                               |                                   |                               |                           |                        |                           |                               |                              |
| I              | 0                             | 2/0                               | 0                             | 4/0                      | 1                      | 1                          | 0                             | 0                            |
| II             | 1                             | 14/2                              | 1                             | 23/2                      | 0                      | 0                          | 4                             | 1                            |
| III            | 3                             | 12/1                              | 0                             | 20/1                      | 0                      | 0                          | 4                             | 0                            |
| IV             | 0                             | 4/0                               | 0                             | 6/0                      | 0                      | 0                          | 1                             | 0                            |
| Differentiationa |                               |                                   |                               |                           |                        |                           |                               |                              |
| Well           | 1                             | 2/0                               | 0                             | 6/0                      | 0                      | 0                          | 2                             | 0                            |
| Moderately     | 3                             | 19/2                              | 0                             | 32/2                      | 1                      | 1                          | 5                             | 0                            |
| Poorly         | 0                             | 11/1                              | 1                             | 15/1                      | 0                      | 0                          | 2                             | 1                            |

*Data not available for some patients.*
Material and Methods

Patients. This study included primary CRC tissue and distant normal mucosa from 356 CRC patients diagnosed at the University Hospital in Linköping and Vrinnevi Hospital in Norrköping. Tissues were collected during primary surgery between 1989 and 2004. Samples from the corresponding normal tissue of the colon or rectum were taken at least 10 cm from the tumor margins. Representative tumor tissues, evaluated by pathologist, were stored for subsequent analyses at −70 °C. Characteristics of the patients are shown in Table 3. The mean age at diagnosis was 72 years. The tumors with better differentiation included well and moderately differentiated tumors, and worse differentiation included poorly differentiated, mucinous or signet-ring cells carcinomas. Information was lacking about tumor differentiation in four patients and recurrence in 169 patients. The study was approved by the Regional Ethical Review Board in Linköping and an informed consent document was signed by participants. The methods were carried out according to the approved ethical guidelines.

Cell culture. The SW480 and SW620 cell lines were obtained from American Type Culture Collection. The cell lines were maintained at 37 °C and 5% CO2 in Eagles MEM (Sigma-Aldrich, St. Louis, MO), supplemented with 10% heat inactivated fetal bovine serum albumin (GIBCO, Invitrogen, Paisley, UK) and 1% L-glutamin (GIBCO). The HCT116 cell line was obtained from the Core cell center (Johns Hopkins University, Baltimore, MD) and was maintained in McCoy’s 5A medium (Sigma-Aldrich) supplemented with 10% heat inactivated fetal bovine serum albumin (GIBCO) at 37 °C and 5% CO2. Cells growing exponentially were harvested when 80% confluence was achieved. All cells were tested for Mycoplasma by using a commercially available PCR kit (PromoKine, Heidelberg, Germany). The morphology and growth rate of all cell lines were controlled during the whole experimental period.

Isolation of DNA and mutation analysis. DNA was isolated from fresh frozen tissue and lysate from cell lines using standard procedures implementing DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The coding region of the MTDH gene was analyzed by using PCR and direct DNA Sanger sequencing in 356 tumors. The exons 1 to 12 and adjacent intronic sequences were amplified using FastStart High Fidelity PCR System (Roche Applied Science, Germany) according to the manufacturer’s instructions. BigDye Terminator v3.1 Ready Reaction Mix (Applied Biosystems, Foster City, CA) was used for sequencing reaction, and separation was performed on ABI 3500 genetic analyzer (Applied Biosystems). The collected data were analyzed by using Sequence analyzer software (Applied Biosystems). Designed primers used for amplification and sequencing analysis are shown in Table 4. Each variant or suspicious fragment was verified by independent PCR amplification and sequence analysis in tumor. Exonic variants that were detected in tumor tissue were analyzed also in the corresponding normal tissue (when available) from the same patients. All detected variants were confirmed by sequencing of forward and reverse strands.

Table 3. Colorectal cancer patients and tumor characteristics. *median survival is 50 months. bdata not available for some patients.
Nomenclature of mutations. Mutations were described according to the nomenclature system recommended by the Human Genome Variation Society (HGVS)\textsuperscript{34}. Designation of the genomic alterations in the MTDH gene is based on the GenBank reference sequences NM_178812. Mutations which were not found in the literature, the Single Nucleotide Polymorphism Database (dbSNP, http://www.ncbi.nlm.nih.gov/SNP/, (accessed in June, 2015)\textsuperscript{35}, or in the Catalogue of Somatic Mutations in Cancer (COSMIC, http://www.sanger.ac.uk/cosmic, accessed in June, 2015)\textsuperscript{36} were considered as novel.

Statistical analyses. Importance of frequent variants was analyzed by using the STATISTICA 10 (StatSoft, Tulsa, OK). The chi-square test was applied to determine the relationship of MTDH variants with clinicopathological variables. Cox’s Proportional Hazard Model was used to test the relationship between the variants and the patient survival. All tests were two sided, and a $P$-value less than 0.05 was considered as significant.

In silico prediction of impact of the variants on protein function. Exonic variants were evaluated by widely used programs for prediction of possible interference with the function, structure or stability of a protein (Supplementary Table 5): Mutation Taster (http://www.mutationtaster.org; Ensembl transcript ENST00000336273, NM_178812; GRCh37/ Ensembl 69), SIFT and GVGD as a part of commercial Alamut 2.0 (Interactive Biosoftware, Roven, France), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/; UniProt peptide Q86UE4), PROVEAN (http://provean.jcvi.org/index.php; Human GRCh37/Ensemble 66) and, MUpro (http://mupro.proteomics.ics.uci.edu).

Table 4. Primer pairs used for PCR amplification and sequence analysis of the MT DH gene.\textsuperscript{a} GenBank reference sequence NC_000008 (chr8:98,656,407–98,742,488; GRCh37).\textsuperscript{b} Underlined primers were preferentially used for sequence analysis.

| Exon | Primers S$'$$' \rightarrow$ T$'$$' | Length of PCR product (bp) |
|------|---------------------------------|---------------------------|
| 1    | F: ACCAATTAACCCCTCCAGC          | 1087                      |
|      | R: CCACTCGGTCTTGAGC             |                           |
|      | SqF1: TTCTCTGACACGCTT          |                           |
|      | SqF2: TCGCTCCCTGACGATCC         |                           |
| 2    | F: AGGTACAGAGGTTAGATTG          | 556                       |
|      | R: AAGGTAAACACAAATTCCACAG       |                           |
| 3–4  | F: GTGTACAGATCTGACTTCT          | 1183                      |
|      | R: AAGGTAGCTACAAATTCCACAG       |                           |
|      | SqF1: ACCCTATCTGCGGAGGAG       |                           |
|      | SqF2: TCAACACTCTTGTTTTAG        |                           |
| 5    | F: GTGGAAGATTTAGACATTTG         | 396                       |
|      | R: ATGTTAGAGGTTGGTGTG           |                           |
| 6    | F: TAAAGGCAATTCTTGGTAGTC        | 547                       |
|      | R: AATCCACCTGGCTCTTAC           |                           |
| 7    | F: ATCTAATGATGTTGCTAGG          | 642                       |
|      | R: TAGGAAGAAGAAACGACATTC        |                           |
| 8    | F: ATGGCACTATAATGTTGG          | 846                       |
|      | R: ATTGGTGTCAGCCCTGTG           |                           |
| 9    | F: ATGACGTAGACACAGTAGAG         | 720                       |
|      | R: ACCAGCAAATCAGGAGCA           |                           |
| 10   | F: AGGAATTCTCCTACCTCCT          | 677                       |
|      | R: TGCTTCTGAACTTCCGAGCT         |                           |
|      | SqF: TCTCTCGACACTCAAGTAGC       |                           |
| 11   | F: AGAGGGCAGGTAGTGGTTAC         | 477                       |
|      | R: TAGCCAGGTGTCCTGTAG           |                           |
| 12   | F: AAGGAGGAGAAGAAGACATAG        | 469                       |
|      | R: TTCCCAAATGGTCTCTCCT          |                           |

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Author Contributions
Study concept and design: S.G., I.T., X.F.S. Acquisition of data: S.G., I.T. Analysis and interpretation of data: S.G., I.T., X.F.S. Statistical analyses: S.G. Contribution of the patient material with clinical data: S.H. Drafting of the manuscript: S.G., I.T., X.F.S. All authors approved the final manuscript.

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