Metadherin (MTDH) Overexpression Significantly Correlates with Advanced Tumor Grade and Stages among Colorectal Cancer Patients

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Research Article

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Abstract

Background

Colorectal cancer is the 4th leading cause of cancer related deaths affecting both men and women worldwide. In the present study, any probable role of MTDH mRNA expression in CRC tumorigenesis was explored using both discovery and validation cohorts.

Methods and results

After prior ethical and biosafety approvals, tumor tissue samples along with their adjacent controls were collected for this study from Pakistani patients diagnosed with colorectal cancer. RNA was isolated using Trizol reagent, followed by cDNA synthesis. Transcript analysis of MTDH was performed by using qPCR. Moreover, genome-wide expression of MTDH was also determined through micro-array data analysis using BRB- Array Tools software. MTDH expression was significantly high in tumor tissue samples ($p < 0.05$) compared to their respective controls. Likewise, results of microarray analysis also revealed overamplification of MTDH in tumor samples as compared to controls. Expression of MTDH was also found to be positively correlated with KI-67 index ($p < 0.05$) and were observed to be significantly upregulated in advance tumor grade ($p < 0.05$) and stage ($p < 0.05$). However, no association of MTDH overexpression with age and gender could be established.

Conclusion

Hence, it can be concluded that MTDH is a core element that plays a pivotal role in colorectal tumorigenesis irrespective of patient's age and gender. Molecular insight into the tumor microenvironment revealed MTDH as a niche, representing distinctive framework for cancer progression, thus, making it an innovative target strategy for colorectal cancer treatment.

Introduction

Colorectal cancer is categorized among top three most common malignancies across the globe. Earlier, incidence of colorectal cancer was frequently observed in countries with high Human development index. However, recent reports have shown that due to environmental changes and sedentary lifestyle, incidence of CRC is gradually mounting in developing countries [1].

Colorectal carcinogenesis is a multi-step process that results due to transcriptional amplification of various oncogenes and activation of assorted biological pathways. Pertinent studies have unveiled three main pathogenic mechanisms i.e., microsatellite instability (MSI), CpG island methylator phenotype (CIMP) and chromosomal instability (CIN) pathway, to be responsible for 80-85% of CRC cases. Activation of these pathways have been linked with accretion of different mutations in several oncogenes and tumor suppressor genes such as SMAD2, BRAF, P53, EGF, MYC and RAS [2]. Being one of the most frequently activated oncogenes, RAS is considered as a prime key player in majority of colon cancer

cases [3]. Interestingly, RAS mediated transcriptional activation of MTDH activates several oncogenic pathways including P13K/Akt, WNT, NF- κ B and MAPK involved in tumorigenesis [4].

Previously, expressional dysregulation of MTDH has been linked with aggressive phenotype and poor prognosis in breast cancer patients [5]. In fact, it is included as salient biomarker among 70 genes in MamaPrint assay designed to estimate tumor recurrence [6]. Elevated expression of MTDH has also been observed in other types of cancers including lung [7], ovarian [8], prostate [9] and rectal cancers [10]. However, the involvement of MTDH in colorectal cancer has not been previously reported in Pakistani patients. Therefore, the present study aims to assess MTDH expression in CRC patients of Pakistan in conjunction with its association with the clinicopathological parameters of the study cohort. Additionally, expressional dysregulation of MTDH is also substantiated using a publicly available dataset to deduce an affirmative conclusion.

Materials And Methods

Sample collection and data retrieval

To serve the purpose of this study, 82 colorectal tumor tissue samples along with their adjacent controls, were collected from Pakistan Institute of Medical Sciences (PIMS) hospital at the time of surgery after obtaining an informed patient consent and ethical approval from the collaborating hospital. Moreover, patient's clinical information and histopathological data were also retrieved in the subsequent follow up.

MTDH expression analysis in the discovery cohort

RNA extraction and cDNA synthesis

RNA extraction was accomplished for each sample using TRIzol reagent (Invitrogen, California, USA) following an already established protocol [11]. The RNA, thus extracted, was quality assessed by measuring absorbance at 260/280nm and 230/260nm using nanodrop spectrophotometer (IMPLEN, Germany). Reverse transcription of extracted RNA was carried out using Revert Aid First Strand cDNA synthesis kit following manufacturer's instructions. GAPDH was used as a housekeeping gene for confirmation of cDNA.

Quantification of MTDH, Ki67 and GAPDH expression

Primers for MTDH, Ki67 and GAPDH were designed using Primer 3 software. Transcript copies of MTDH, Ki67 and GAPDH were quantified by means of SYBR green (Solis Biodyne, Estonia) qPCR technology (Applied Biosystem, USA). Differential gene(s) expression was quantified from Ct values by exploiting Livak method [12].

Validation of MTDH expression at protein level

MTDH expression at protein level was assessed in normal as well as in colon tumor samples by means of immunohistochemistry-based expression profiling in an attempt to validate its abundance in CRC patients using protein atlas.

Validation of MTDH expression using microarray data

Involvement of MTDH was also assessed in genome wide expression in two independent datasets. Dataset with ID (GSE21510) was retrieved from Gene Expression Omnibus (GEO) NCBI, that consisted of 129 samples, including both tumor and controls. Data set under the ID (GSE2509) was used to assess role of MTDH in colon cancer cell metastasis. Differential expression was compared among SW480 (primary) and SW620 (metastatic) colon carcinoma cells. Microarray analysis of this raw data was performed using BRB-Array Tools software. Considering the heterogeneous nature of CRC, molecular characterization of MTDH high vs MTDH low tumors was also performed in order to see if there was any difference at molecular level between the tumors signifying normal and dysregulated MTDH gene. In order to assess the differential expression of genes between assorted classes, “class comparison” (one of the objectives of BRB Array Tools) was performed at fold change threshold of 1.5, $p < 0.05$ and stringent false discovery rate (FDR).

Statistical evaluation of data

Non-parametric methods of testing i.e., Wilcoxon Signed Rank Test, Kruskal Wallis, ANOVA and Mann-Whitney were used for statistical analysis of data obtained after qPCR analysis. Correlation of MTDH with other genes was analyzed by means of Spearman Rank Correlation Coefficient test using Graph-pad Prism Version 5.0. P-value less than 0.05 was considered as statistically significant.

Results

MTDH levels were significantly higher in colon cancer tissues in comparison to normal

The transcript levels of MTDH were found to be significantly higher in tumors compared to their adjacent controls in the Pakistani CRC cohort ($P < 0.05$) (Figure 1). In-line with these findings, validation cohort also showed a similar trend in MTDH expression. Microarray data analysis the data set GSE21510 showed a total of 13,437 genes, including MTDH, to be differentially expressed in colorectal cancer at nominal significance level of 0.05 and fold change 1.5, as depicted in the Figure 2. Validation of MTDH expression also revealed its upregulation at protein level in colorectal patients, as shown in Figure 3.

High MTDH increases tumor proliferation

Correlation of MTDH with KI-67 (proliferative marker) was observed to be strongly positive and statistically significant ($p < 0.05$), with r-Value of 0.4.

Correlation of MTDH with different clinico-pathological parameters

MTDH Expression was significantly upregulated in advance stage, high tumor grade (Figure 1). Similar results were found in validation cohort where elevation in number of polysome associated MTDH mRNA was observed in metastatic colorectal cancer cell lines (SW620) as compared to the cell lines obtained from primary tumor site (SW480), as shown in Figure 4. Statistically significant MTDH overexpression was also observed in mucinous adenocarcinoma, and in caecum when compared against rectum (Figure 1). However, rectal tumors showed relatively higher MTDH expression in comparison to colon ($P<0.05$) (Fig. 1).

Association of MTDH with demographic parameters

Considering age as one of the risk factors for developing CRC, amplification level of MTDH was also analyzed in different age groups based on cohort’s mean age calculated to be 48 years. Higher MTDH mRNA levels were observed in patients greater than 48 years. Furthermore, gender-based analysis of data showed that MTDH expression of MTDH was higher in males as compared to females. However, no statistical significance of age and MTDH transcript levels could be established in either gender or the two age groups.

Differential expression of MTDH using microarray data analysis

Molecular characterization of MTDH high vs MTDH low tumors revealed significant number of differentially expressed genes in MTDH high tumors. Results of the study showed, a high number of membrane related proteins, growth factors, receptors and receptor associated kinases, e.g., EGF, MME, SPC25, EREG, AQP1, GGH, SNX16, PPBP, TFF1, HPGD, PIPOX etc., to be dysregulated in MTDH high tumors. Moreover, 32 percent of genes in MTDH high vs low DEG set belonged to chromosome number 8 (Table 1), which is also a locus for MTDH gene, as displayed in Figure 5. These findings indicate that tumors emerging as a result of MTDH dysregulation may follow a different pathogenic pathway in contrast to those developing under the influence of any other gene/group of genes.

Table 1: Highest number of dysregulated genes in MTDH high vs low tumor comparison belonged to chromosome 8

Chromosome	Chr 8 gene/ total DEG set	% of Chr 8 genes in DEG set	PValue	Genes
CHROMOSOME 8	52/163	32	1.09E-32	ENY2, PDP1, NBN, NDUFAF6, OSGIN2, DPY19L4, TMEM64, MTFR1, CCNE2, DCAF13, FAM49B, NUDCD1, NSMCE2, SLC5A1, OXR1, TATDN1, RNF170, ATP6V1C1, TMEM67, SLC25A32, C8ORF59, DSCC1, POLR2K, ZBTB10, OTUD6B, SNX16, LRRCC1, PRKDC, RRM2B, ZFAND1, MRPL13, MRPL15, RB1CC1, MTERF3, TBC1D31, LAPTM4B, SBSPON, UTP23, MRPS28, SAMD12, C8ORF76, GGH, ATAD2, C8ORF33, TMEM55A, CSPP1, PLEKHF2, ANKRD46, POP1, RAD54B, TCEB1, FABP5

Discussion

Metadherin (MTDH), a multifunctional protein, is encoded by MTDH gene located at q22 arm of chromosome 8. Oncogenic H-Ras has been known to play prominent role in MTDH overexpression through activation of PI3Akt signalling cascade [13]. In this regard, the current study was conducted to investigate expressional dysregulation of MTDH in colorectal cancer cohort of Pakistan and correlate it with different clinico-pathological parameters. The findings, thus obtained, were validated through high-throughput expression profiling of MTDH using selected datasets. Metastatic role of MTDH in different cell lines and its synergistic interaction with several cancer genes was also studied.

High MTDH expression (2 folds) in tumor tissue samples compared to their adjacent controls in the discovery cohort (Figure 2) as well as in the validation cohort (Figure 3) may possibly be attributed to MTDH gene locus positioned at Chromosome 8 [13]. In this context, similar observations have been made for breast [14], ovarian [8], gastric [15] and non-small lung cancer [7]. Yet another study also reported that 'q' arm of chromosome 8 was associated with evolvement and promotion of pulmonary blastoma [16]. Similarly, acquisition of 8q22 locus was directly associated with overexpression of MTDH and provocation of many biological functions in breast carcinoma [17]. Hence, there might be occurrence of an upstream event on chromosome number 8 that results in dysregulation of various oncogenes and tumor suppressor genes.

Additionally, present study characterized tumor microenvironment based on MTDH expression, whereby, several proteins in different cellular compartments, including various membrane proteins (FLRT3, GPR143, TMEM67, EPHA4, DRB4), and AQP1 (an intrinsic membrane pore protein) were found to be dysregulated in MTDH high tumors. As reported, AQP1 protein triggers invasion and metastasis of cancerous cells in mouse melanoma cell lines and colon cancer HT20 cell lines [18]. Moreover, many secretory granules including TFF3, CPA3, RAB27B and GAL, that are known to amplify tumor cell proliferation, invasion, migration and cell survival in colon prostate, breast, gastric and cervical cancers, were also dysregulated in MTDH high tumors as compared to the tumors with low MTDH expression [19].

Moreover, important solute carriers such as SLC28A3, differentially expressed in tumors with MTDH upregulation. Role of a SLC28A3 variant has also been defined in prostate cancer progression and metastasis [20].

Previously, role of MTDH in regulation of cancer hallmarks through activation of various downstream signalling pathways has also been established. However, limited studies have addressed the increase in MTDH mRNA copy number in tumor cells, hence, necessitating this study. Differential expression of EGF gene in MTDH high tumors in two different cellular classes including whole membrane and late endosomal membrane was also revealed in this study, which mediates various downstream mitogenic pathways together with P13/Akt signalling [21]. Hence, perpetual mutational activation of EGFR receptor might serve as a possible reason for MTDH overexpression in tumor cells.

Notably, 32 percent of dysregulated genes in MTDH high tumors were present on chromosome number 8 as compared to MTDH low tumors (Table 1), which may be due to the presence of c-Myc on the same chromosome which induces expression of various genes by binding to E-box elements in promoter region of almost 15- 20 percent of genes, genome-wide [4]. Similar findings concerning c-Myc induction have been reported for melanoma cancer cells, whereby, activation of Ras/P13AKT/ERK pathway (responsible for upregulation of MTDH gene) was directly linked with the inducibility of c-Myc gene in the presence of ADI-PEG20 [22].

Significant correlation between MTDH and KI-67 index also validates role of MTDH in tumor cells proliferation, which coincides with the results reported in bladder cancer and breast carcinoma [23]. These findings suggest that MTDH plays important role in development and progression of CRC where higher transcript levels of MTDH at early stage could be considered as a monitory point in metastatic transformation of colorectal tumors.

In-line with previous studies, higher copy number of MTDH were present in advance stage carcinoma and in anaplastic tissues [10]. In another study, it was reported that MTDH- induced liver metastasis of colorectal cancer. Moreover, it was also illustrated that higher expression of MTDH may lead to poor prognosis in metastatic colorectal cancer [24]. These reports are consistent with the findings obtained in the current study. High copy number of MTDH-mRNA was observed in patients exhibiting lymph node invasion in Pakistani study cohort. Assessment of translational status of MTDH-mRNA using two different cell lines (Figure 4) also showed that increased MTDH mRNA was associated with polysomes in metastatic CRC cell lines (SW620) as compared to primary CRC cell lines (SW480), suggesting that metastatic cells have more MTDH protein produced as compared to cancer cells at their primary site.

Characterization of MTDH high tumor microenvironment also indicated the number of differentially expressed genes that play key role in development and progression of carcinogenesis. So, we can hypothesize that, this involvement of MTDH in metastasis could be due to its synergistic effect on different oncogenes involved in tumor progression. As, wang *et al*, exposed in their study that MTDH intensifies the metastatic effect of tumor cells by up regulating the expression of Matrix Metallo Proteases (MMPs) [5]. Another study also showed that overexpression of MTDH augments the

transcription of several oncogenes including cyclin-D1 and c-Myc by upregulating the expression of transcription factor LEF1 [25]. Furthermore, high MTDH transcript levels were observed in mucinous adenocarcinoma as compared to conventional adenocarcinoma ($P<0.05$), in-line with the reports published already [10].

Due to involvement of multiple parts of colon and rectum, localization of tumor in colorectal cancer is always a matter of concern. It is reported that tumors of rectum are more likely to emerge from CIN pathway and carcinomas of colon originate from MSI pathway [26]. Likewise, current study also endorsed these findings for expression of MTDH was higher in rectal cancer as compared to colon cancer, consistent with reports published by Gnosa and colleagues [10]. This heterogeneity and differential expression of MTDH may be attributed to anatomical, histological and etiological differences among different sites of colon and rectum. Moreover, we can also speculate that differential expression of MTDH in different parts of colon and rectum may act as a diagnostic marker and potential treatment option for metastatic colorectal cancer.

Conclusion

In conclusion, this study suggests that MTDH plays a crucial role in colorectal cancer initiation and progression, thus, can be exploited as a potential biomarker for screening purposes. Moreover, these findings also demonstrated that tumors with high MTDH expression might follow a different pathway as compared to MTDH low tumors, recommending MTDH to be a potential therapeutic target in future cancer treatment. However, further studies are required to identify its role in targeted gene therapy for CRC treatment.

Abbreviations

CRC	Colorectal cancer
MTDH	Metadherin
MMPs	Matrix Metallo Proteases
CIN	Chromosomal Instability
MSI	Microsatellite Instability

Declarations

Data availability

Data used in this study can be obtained from corresponding author on special request. Data sets used for in-silico study can be assessed through GEO- NCBI website under the accession numbers provided in this study.

Ethical approval

In order to conduct this study, ethical consent was obtained from the Ethical Committees of Pakistan Institute of Medical Sciences (PIMS) hospital and COMSATS University Islamabad.

Competing interests

The authors of the study have no conflict of interest to declare.

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Consent to participate

Informed consents of all participants (patients) were obtained to conduct this study.

Consent for publication

All authors of the study showed agreement to publish this study in Molecular Biology Reports journal.

Author's contribution

A.S drafted the project, executed wet lab and in- silico experiments, performed micro array data analysis and statistical data analysis, prepared tables and figures and wrote the research paper. N.e.S contributed equally and performed lab experiments, micro-array data analysis study and revised the manuscript. S.K.R helped in data analysis and preparation of table and figures. J.Q revised and edited the manuscript. S.H.W and T.K performed surgeries and helped in sample collection. M.F.A.M designed and supervised the study and approved the final draft of the manuscript.

Code availability

Not applicable

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Figures

Figure 1

Differential Expression of MTDH in Colorectal Cancer Cohort and its Expressional Correlation with Various Clinico-pathological Parameters.

Figure 2

Graphic representation of differentially expressed behaviour of MTDH in colorectal cancer. (a) Differential expression of MTDH in colorectal cancer vs normal intestinal mucosa. (b) Differentially expressed behaviour of MTDH in colorectal cancer with respect to different stages (c) Heat map showing differential expression of MTDH in colorectal cancer cohort (tumor vs normal).

Figure 3

Validation of MTDH Protein Expression in (a) Normal Colon and (b) Colorectal Cancer Patients (<https://www.proteinatlas.org/>).

Figure 4

Translational status of MTDH mRNA in primary SW480 and metastatic SW620 colon carcinoma cell lines (GSE2509).

Figure 5

Differential expression of genes (DEG) in MTDH high vs low tumors. (a) Heatmap of differentially expressed genes (DEG) in MTDH high vs Low classes. (b) Chromosomal distribution of entire gene set (Red) and DEG subset (Blue) in MTDH High vs Low class comparison.