**Hypermethylation and Decreased Expression of *TMEM240* are Potential Early-Onset Biomarkers for Colorectal Cancer Detection, Poor Prognosis and Early Recurrence Prediction**

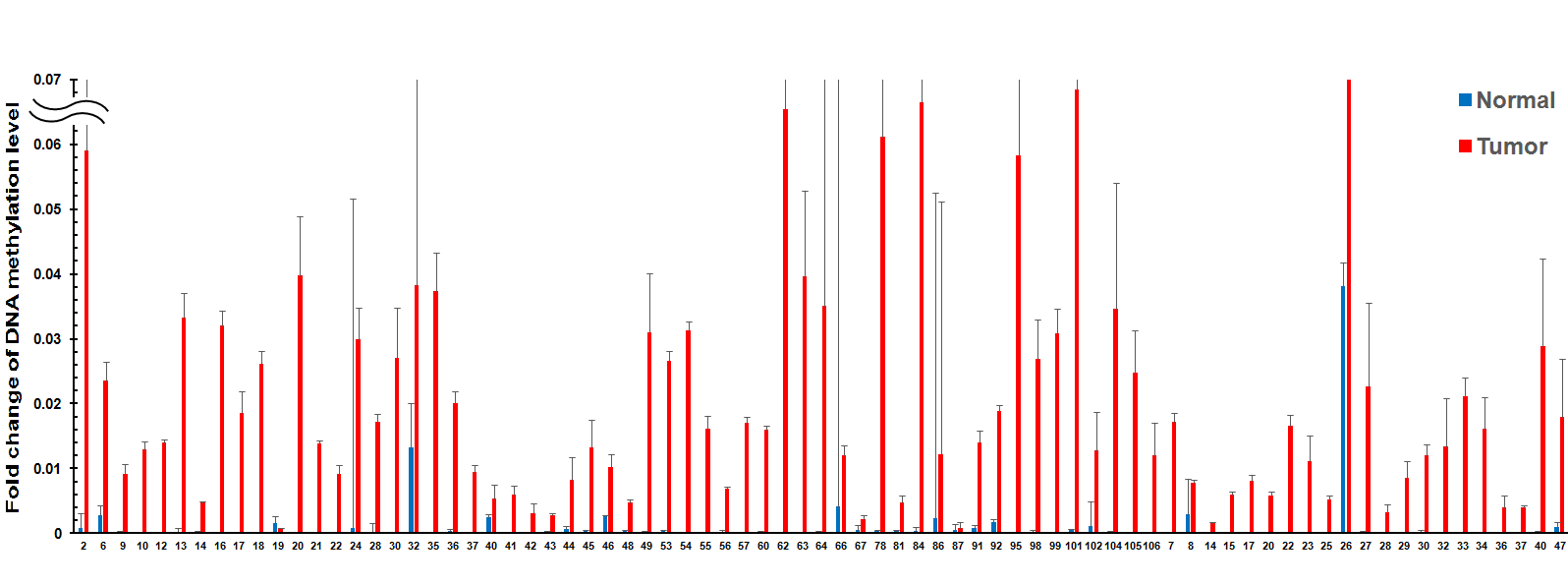
**Supplementary figures**

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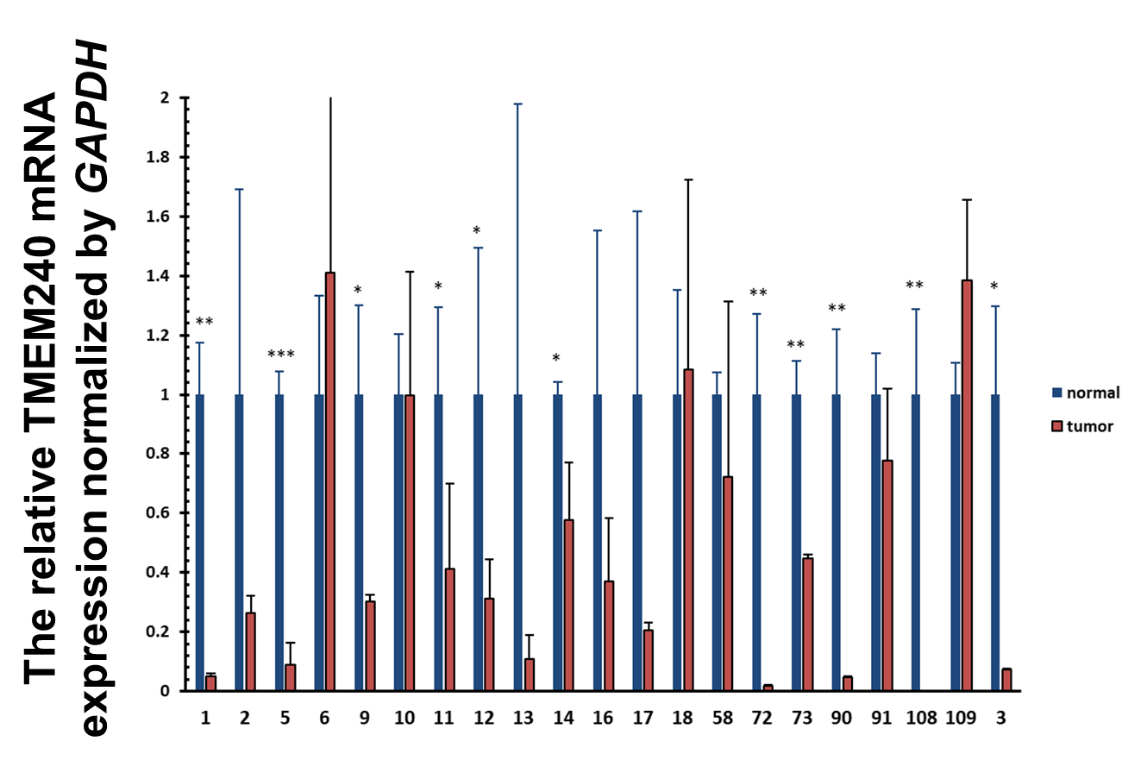
**Figure S1.** Differentially methylated CpG heatmap of *TMEM240* in paired colon cancer, esophageal cancer, gastric cancer, pancreatic cancer, rectal cancer, and liver cancer tissues. Methylation levels (average β values) at differentially methylated loci were identified using an Illumina Methylation 450K array-based assay.

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**Figure S2.** *TMEM240* mRNA expression was analyzed in two colon cancer cell lines and two human normal colon tissues.



**Figure S3.** Representative figures of *TMEM240* methylation levels determined by QMSP in 78 adjacent normal colon tissues and 78 CRC tumors. Experiments were performed with three technical replicates.



**Figure S4.** Representative figures of *TMEM240* mRNA expression determined by gene specific probe based real-time RT-PCR in 21 adjacent normal colon tissues and 21 CRC tumors. Experiments were performed with three technical replicates. Data are presented as the mean ± SD, \* *p* ≤ 0.05, \*\* *p* ≤ 0.01, \*\*\* *p* ≤ 0.001. A *t*-test was used to calculate group differences in all experiments. Experiments were performed with at least two biological duplicates and three technical replicates.

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**Figure S5.** The Kaplan-Meier survival curves for (A) overall survival in Taiwanese colorectal carcinoma (CRC) patients and (B) cancer-specific survival in Taiwanese females are shown. *TMEM240* was considered to have low expression when the expression level in CRC tumors was 5-fold lower than that in normal tissues.

**Table S1. *TMEM240* mRNA expression and promoter hypermethylation in relation to the clinical parameters of the TCGA CRC dataset.a**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Characteristics** | | **Total n** | ***TMEM240* mRNAb**  **Low n (%) High n (%)** | | | | | **Total n** | ***TMEM240* Methylationc**  **Low n (%) High n ( %)** | | | | | | |
|  |  |  |  | | |  |  | | |  | | | |
| **Overall** | | 456 | 389 | (85.3) | | 67 | (14.7) | 293 | 48 | (16.4) | 245 | | (83.6) | |
| **Age** | |  |  |  |  | |  |  |  |  | |  | |  | |
|  | < 65 | 184 | **150** | **(81.5)** | **34** | | **(81.5)0.090** | 136 | **31** | **(22.8)** | | **105** | | **(77.2)0.008** | |
|  | > 65 | 261 | **228** | **(87.4)** | **33** | | **(12.6)** | 146 | **16** | **(11.0)** | | **130** | | **(89.0)** | |
| **Race** | |  |  |  |  | |  |  |  |  | |  | |  | |
|  | Asian | 11 | **11** | **(100.0)** | **0** | | **(0.0)0.265** | 11 | **2** | **(18.2)** | | **9** | | **(81.8)0.970** | |
|  | Black or African American | 52 | **46** | **(88.5)** | **6** | | **(11.5)** | 51 | **8** | **(15.7)** | | **43** | | **(84.3)** | |
|  | White | 211 | **177** | **(83.9)** | **34** | | **(16.1)** | 201 | **34** | **(16.9)** | | **167** | | **(83.1)** | |
| **Sex** | |  |  |  |  | |  |  |  |  | |  | |  | |
|  | Male | 211 | **176** | **(83.4)** | **35** | | **(16.6)0.391** | 130 | **25** | **(19.2)** | | **105** | | **(80.8)0.285** | |
|  | Female | 234 | **202** | **(86.3)** | **32** | | **(13.7)** | 152 | **22** | **(14.5)** | | **130** | | **(85.5)** | |
| **Tumor Type** | |  |  |  |  | |  |  |  |  | |  | |  | |
|  | Adenocarcinoma | 383 | **330** | **(86.2)** | **53** | | **(13.8)0.113** | 245 | **38** | **(15.5)** | | **207** | | **(84.5)0.180** | |
|  | Mucinous | 60 | **47** | **(78.3)** | **13** | | **(21.7)** | 37 | **9** | **(24.3)** | | **28** | | **(75.5)** | |
| **Tumor Stage** | |  |  |  |  | |  |  |  |  | |  | |  | |
|  | I | 75 | **67** | **(89.3)** | **8** | | **(10.7)0.323** | 43 | **2** | **(4.7)** | | **41** | | **(95.3)0.127** | |
|  | II | 176 | **153** | **(86.9)** | **23** | | **(13.1)** | 112 | **22** | **(19.6)** | | **90** | | **(80.4)** | |
|  | III | 122 | **99** | **(81.1)** | **23** | | **(18.9)** | 79 | **14** | **(17.7)** | | **65** | | **(82.3)** | |
|  | IV | 61 | **50** | **(82.0)** | **11** | | **(18.0)** | 38 | **8** | **(21.1)** | | **30** | | **(78.9)** | |
| **Tumor Size** | |  |  |  |  | |  |  |  |  | |  | |  | |
|  | T0-T1 | 12 | **11** | **(91.7)** | **1** | | **(8.3)0.509** | 8 | **0** | **(11.8)** | | **8** | | **(100.0)0.199** | |
|  | T2-T4 | 433 | **367** | **(84.8)** | **66** | | **(15.2)** | 274 | **47** | **(17.2)** | | **227** | | **(82.8)** | |
| **Regional lymph nodes metastasis** | | | |  |  | |  |  |  |  | |  | |  | |
|  | N=0 | 266 | **234** | **(88.0)** | **32** | | **(12.0)0.028** | 166 | **26** | **(15.7)** | | **140** | | **(84.3)0.566** | |
|  | N>1 | 178 | **143** | **(80.3)** | **35** | | **(19.7)** | 115 | **21** | **(18.3)** | | **94** | | **(81.7)** | |
| **Distant metastasis** | | | |  |  | |  |  |  |  | |  | |  | |
|  | M=0 | 324 | **277** | **(85.5)** | **47** | | **(14.5)0.359** | 186 | **30** | **(16.1)** | | **156** | | **(83.9)0.553** | |
|  | M>1 | 63 | **51** | **(81.0)** | **12** | | **(19.0)** | 40 | **8** | **(20.0)** | | **32** | | **(88.0)** | |
| **MSI** | |  |  |  |  | |  |  |  |  | |  | |  | |
|  | No | 80 | **68** | **(85.0)** | **12** | | **(15.0)0.168** | 76 | **15** | **(19.7)** | | **61** | | **(80.3)0.105** | |
|  | Yes | 11 | **11** | **(100.0)** | **0** | | **(00.0)** | 11 | **0** | **(0.0)** | | **11** | | **(100.0)** | |
| **Kras mutation** | |  |  |  |  | |  |  |  |  | |  | |  | |
|  | No | 361 | **304** | **(84.2)** | **57** | | **(15.8)0.355** | 200 | **28** | **(14.0)** | | **172** | | **(86.0)0.038** | |
|  | Yes | 47 | **42** | **(89.4)** | **5** | | **(10.6)** | 45 | **12** | **(26.7)** | | **33** | | **(73.3)** | |
|  | |  |  |  |  | |  |  |  |  | |  | |  | |
|  |  |  |  |  |  | |  |  |  |  | |  | |  | |
|  | 1. These results were analyzed by Pearson's *X2* test. Significant *p* values are indicated by superscripts. For some categories, the number of samples (*n*) was lower than the overall number analyzed because clinical data were unavailable for these samples. 2. When the *TMEM240* expression level determined by RNA sequencing analysis of CRC tumors from the TCGA dataset was less than the median *TMEM240* expression level in adjacent normal colon tissues, a sample was considered to have low expression. 3. When the *TMEM240* methylation level (ΔAvg\_β), as determined with the Illumina Infinium HumanMethylation450 BeadChip array, in CRC tumors from the TCGA dataset was higher than 0.25, a sample was considered to have hypermethylation. | | | | | | | | | | | | | | | |

**Table S2 List of primers sequences and their reaction conditions used in the present study**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Gene** | **primer** | **5’→3’sequences** | **Application** | **Size**  **(bp)** | **Tm ()** |
| *GAPDH* | Forward  Reverse  Probe | AGCCACATCGCTCAGACAC  GCCCAATACGACCAAATCC  Roche Universal Probe # 60 | RT-PCR  Real-time | 66 | 60 |
| *TMEM240* | Forward  Reverse  Probe | ATCGCGTGCTTGATGGAC  GGATCACGTAGTGGATATGGTG  Roche Universal Probe # 88 | RT-PCR  Real-time | 125 | 60 |
| *BACTIN* | Forward  Reverse | TGGTGATGGAGGAGGTTTAGTAAGT  AACCAATAAAACCTACTCCTCCCTTAA | MSP-M | 132 | 60 |
| *TMEM240* | Forward  Reverse  Probe | TTTTTCGTTTATTATTACGATCGAC  CAAAAAAACGCTAACCTCTACG  TTTAGAATTATGAAGATTATGGTGTTC | MSP-M | 177 | 60 |