Analysis of a novel protein in human colorectal adenocarcinoma

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Abstract. Colorectal adenocarcinoma (CRC) is the third most common type of cancer worldwide with a low 5-year survival rate. The present study aimed to investigate the structure and function of a novel protein identified from human colorectal adenocarcinoma (CRC). A differentially expressed sequence tag (GenBank accession number, ES274081) was collected from GenBank. Bioinformatics tools were employed to obtain the sequence of the full-length cDNA in order to localize the open reading frame and to predict the protein sequence. Mass spectrometry was used to analyze the structure of this novel protein and western blot analysis was used to confirm the expression of this protein in human CRC tissue samples. The full-length cDNA was composed of 4,283-bp nucleotides and the sequence information was obtained (GenBank accession number, NM_001013649). The corresponding protein molecule contained 165 amino acids, with a monoisotopic molecular weight of 18.6033 kDa and an isoelectric point of 8.43, determined by mass spectrometry. The protein structure and its function in adenocarcinoma were further explored. In the present study, a novel protein, which may be involved in nuclear signal transduction, was identified using bioinformatics, mass spectrometry and western blot analysis.

Introduction

Colorectal adenocarcinoma (CRC) is the third most common type of cancer worldwide accounting for 8.9-9.4% of all cancer cases (1,2). The 5-year survival rate for poorly differentiatied CRC is 29% (3).

Development of human CRC is a multistep process, involving numerous pathological changes in gene expression and protein function. In a previous study, a cDNA subtraction library was established and from this, 86 differentially expressed sequence tags in human CRC were identified using the suppression subtractive hybridization technique combined with cDNA microarray (4). Among these newly identified differentially expressed tags, mRNA expression of ES274081 was observed to be downregulated in human CRC by quantitative real time-polymerase chain reaction (5), indicating a potential role in the development of human CRC. In the present study, to explore the function of NM_001013649, the corresponding protein, HCRCN81, was identified using bioinformatic tools, mass spectrometry and western blot analysis.

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Key words: novel protein, human colorectal adenocarcinoma, bioinformatics

Materials and methods

Cloning of differentially expressed tags in human CRC. Differentially expressed tags were identified from a cDNA subtraction library, using the suppression subtractive hybridization technique combined with cDNA microarray (GenBank accession number, ES274081). Internet sources and bioinformatics analysis software packages are shown in Table I.

Bioinformatical analysis. Using the website, http://www.ncbi.nlm.nih.gov/unigene, a search was performed on the accession number (ES274081) and the sequence information of a 4,283-bp cDNA molecule (GenBank accession number, NM_001013649) was obtained. Using the open reading frame (ORF) finder tool provided by NCBI, the initiation codon was predicted according to the principle that the initiation codon in the Kozak sequence must be suitable for translation initiation (6). According to the Chou-Fasman prediction method of the secondary structure of proteins, 41 amino acids are capable of forming 2 helices. The hydrophobic portion of NM_001013649 was analyzed with ProtScale and the α-helix and β-sheet structures were predicted using PredictProtein. In addition, the coded amino acid sequence was analyzed using BLAST, CPHmodels, SMART, Pfam and Motif Scan.

Mass spectrometric analysis. Mass spectrometry (Proevolab, Beijing, China) was used to confirm the predicted amino acid sequence of NM_001013649 (7). Briefly, the protein sample was digested on ice with trypsin (0.01 μg/μl; 10 μl). Following removal of trypsin, the digested sample was incubated with an ammonium bicarbonate solution (5 μl; 25 mM) at 37˚C overnight. The incubated sample (2 μl) was mixed. The liquid gradient was set at 136 min and the MS acquisition was set at 110 min. For the first-round scan, Fourier transform mass spectrometry was used with a range of 400-1,500 Da. For the second-round scan, linear ion trap quadrupole was used.

Table I. Internet sources.

Internet Sources
http://www.ncbi.nlm.nih.gov/unigene
http://blast.ncbi.nlm.nih.gov/Blast.cgi
http://www.ncbi.nlm.nih.gov/gorf/gorf.html
http://web.expasy.org/protscale/
http://www.predictprotein.org/
http://www.cbs.dtu.dk/services/NetNGlyc/
http://www.cbs.dtu.dk/services/NetPhos/
http://www.cbs.dtu.dk/services/CPHmodels/
http://smart.embl-heidelberg.de/
http://hits.isb-sib.ch/cgi-bin/PFSCAN
http://web.expasy.org/peptide_mass

Western blot analysis. Tissue samples were obtained from two patients: a 74-year-old female patient with rectal tubular villous carcinoma, with a complication of moderate epithelial hyperplasia and a 76-year-old male patient with rectal adenocarcinoma. Tumor and normal tissue located 5 cm from the tumor tissues were collected to detect the expression of the target protein using 12% SDS-PAGE. For each reaction, 30 μg samples were used and the antibody serum was diluted by 1:500. The antibody was made to order by CWBio, Beijing, China (patent number: 201210445862.6). Informed consent was obtained from the patients and the study was approved by the Ethics Committee of Sichuan University.

Results

Bioinformatic analyses. Using the NCBI database, a 4,283 bp target full-length cDNA located in chromosome 2, ORF 68 (2p11.2) was obtained. An initiation codon (ATG) and a termination codon (TGA) were located at the 73-75 and 571-573 nucleotides of this sequence, respectively, which defined the longest ORF in this sequence, representing 166 amino acids (Fig. 1).

The corresponding protein molecule contained 166 amino acids with a comparative MW of 18,750.9 kDa and a pI of 8.44. Protein hydrophilic analysis (Fig. 2) revealed the presence of a hydrophilic fragment, in the region of 80-90 amino acids and a hydrophobic fragment in the region of 115-125 amino acids. Using the tool NetNGlyc, no *O*- or *N*-linkage glycosylation locus was observed in this sequence. Using NetPhos, potential phosphorylation sites were observed at the following amino acid residues: 39, 56, 78, 86, 89, 92, 93, 94, 95, 98, 103, 116, 120, 134, 138, 141 and 164 (Fig. 3). In addition, no transmembrane region or orientation was identifed in this sequence. The results of the coiled-coil analysis is shown in Fig. 4. β-turn analysis indicated that a β-turn may be present near amino acid residues 6, 13, 60, 93, 131 and 145 (Fig. 5).

HCRCN81. In the predicted secondary structure of the HCRCN81 protein, 56 amino acid residues were involved in the formation of α-helices, including the 25-31, 38-54, 86-88, 130-139 and 145-163 residues, accounting for 33.73% of the

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 73 atggaggcggggccgcatccccggccggggcactgctgcaagcct
    M  E  A  G  P  H  P  R  P  G  H  C  C  K  P
118 gggggggcggctggacatgaaccacggcttcgtgcaccatatccga
    G  G  R  L  D  M  N  H  G  F  V  H  H  I  R
163 cggaaccagatcgctcgggacgactatgacaagaaggtgaagcag
    R  N  Q  I  A  R  D  D  Y  D  K  K  V  K  Q
208 gcggccaaggagaaggtgaggaggcggcacacgcccgcgccgacg
    A  A  K  E  K  V  R  R  R  H  T  P  A  P  T
253 cggccccgcaagccagacctgcaggtgtacctgccgcgacaccga
    R  P  R  K  P  D  L  Q  V  Y  L  P  R  H  R
298 gatgtctctgcccacccacgcaacccagactatgaagagtccggt
    D  V  S  A  H  P  R  N  P  D  Y  E  E  S  G
343 gaaagcagcagtagtggaggctctgagctggagccttctggccat
    E  S  S  S  S  G  G  S  E  L  E  P  S  G  H
388 cagctcttctgcttagaatacgaggcagacagtggagaggtcaca
    Q  L  F  C  L  E  Y  E  A  D  S  G  E  V  T
433 tcagttatcgtctatcagggtgatgacccaggaaaggtgagtgag
    S  V  I  V  Y  Q  G  D  D  P  G  K  V  S  E
478 aaggtgtcggcacacacgcctctggatccacccatgcgagaagcc
    K  V  S  A  H  T  P  L  D  P  P  M  R  E  A
523 ctcaagttgcgtatccaggaggagattgcaaagcgccagagccaa
    L  K  L  R  I  Q  E  E  I  A  K  R  Q  S  Q
568 cactga 573
    H  *
```

Figure 1. ORF finder.

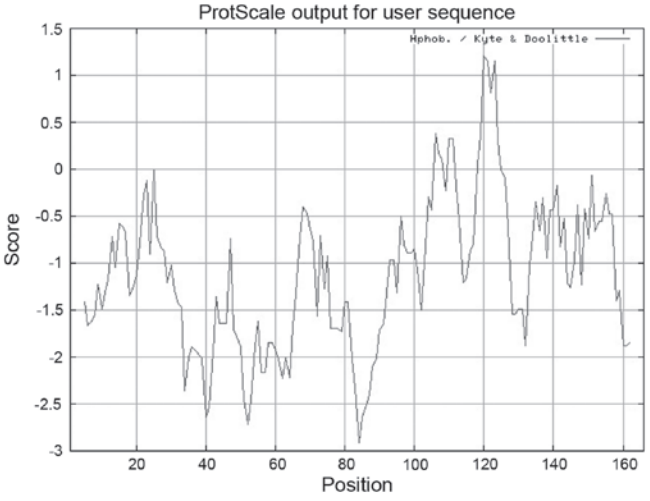

Figure 2. Hydrophobic analysis.

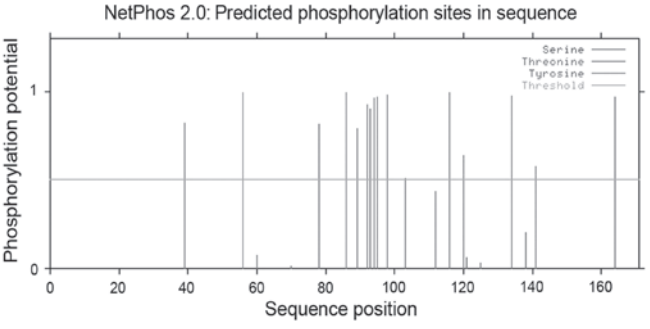

Figure 3. Phosphate analysis.

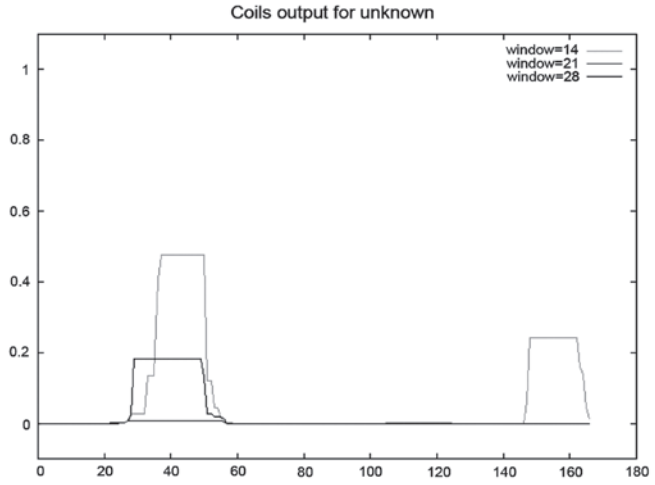

Figure 4. Coiled-coil analysis.

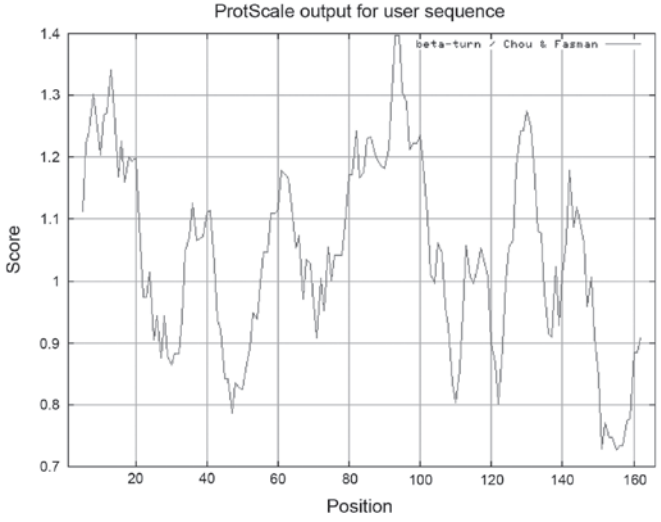

Figure 5. β-turn analysis.

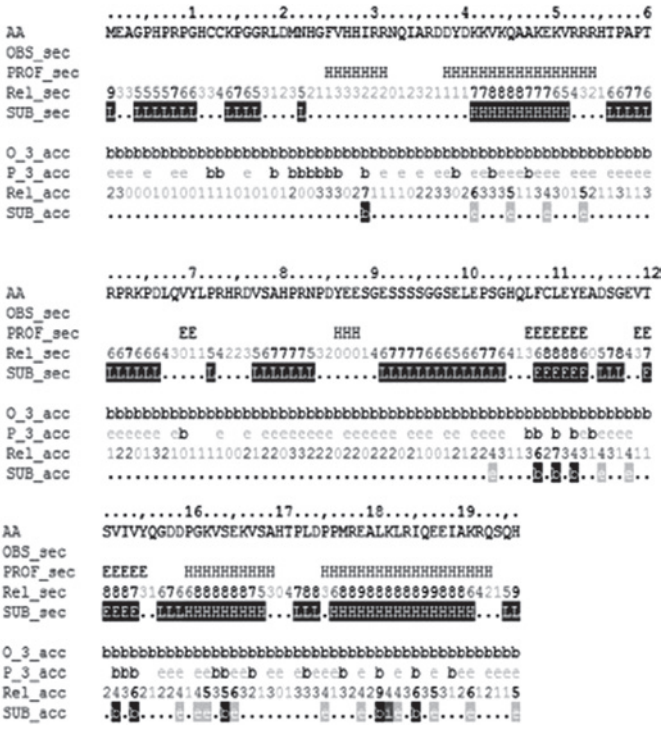

Figure 6. Secondary structure analysis.

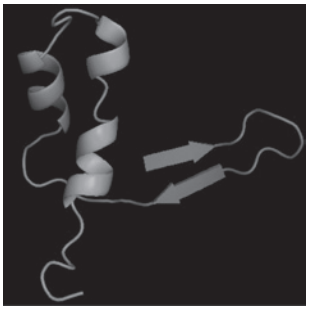

Figure 7. Tertiary structure.

entire protein sequence. By contrast, 16 amino acids were involved in the formation of β-sheets, including the 69-70, 107-113 and 119-125 amino acid residues, accounting for 9.64% of the protein sequence (Fig. 6). Using the tool CPHmodels (http://www.cbs.dtu.dk/services/CPHmodels/) provided by the Center for Biological Sequence Analysis (Lyngby, Denmark) the tertiary structure of this protein was predicted (Fig. 7).

In addition, the localization pattern of the protein, HCRCN81, was explored. Using SignalP, no signal peptide of 1-70 amino acids was identified (Fig. 8). Utilizing TargetP, it was noted that there was a reduced possibility of the protein, HCRCN81, to be localized in mitochondria, endoplasmic reticulum or other components of the secretory pathway. Analysis using the tool NetNES revealed the presence of a leucine-rich nuclear export signal (NES) at amino acid residues 147-155 of the protein, HCRCN81 (Fig. 9).

Analysis of the protein sequence using SMART and Pfam (8), revealed two areas with low complexity at residues 52-65 and 87-104 of HCRCN81. This protein is a member of the UPF0561 family and shares a domain containing 1-126 amino acids with the UPF0561 family members. Using SMART, three regions in HCRCN81 that may be homologs of other known structures were identified: i) 11-39 amino acids shared with SCOP: d1fn9a (E-value 2.20e+00), which was present in the outer capsid protein σ3 (9); ii) 90-108 amino acids shared with SCOP: d1eg3a2 (E-value 7.30e+00), which was present in the EF-hand domain (10); iii) 110-145 amino acids shared with SCOPL: d1qlma_(E-value 1.10e+00), which was present in methenyltetrahydromethanopterin cyclohydrolase (11).

Analysis using Motif Scan revealed cAMP- and cGMP-dependent protein kinase phosphorylation sites at residues 53-56 and 161-164; casein kinase II phosphorylation sites at residues 98-101 and 141-144; protein kinase C phosphorylation sites at residues 134-136; tyrosine kinase phosphorylation sites at residues 63-70 and bipartite nuclear localization signal at residues 30-44.

Using the tool, PeptideMass, it was observed that HCRCN81 may be digested into peptides, the of which sequences are shown in Fig. 10 (selected 500-3,000 kDa).

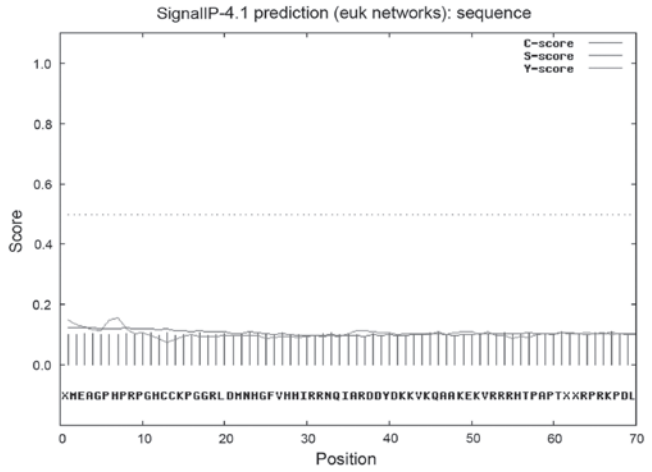

Figure 8. SignalP result.

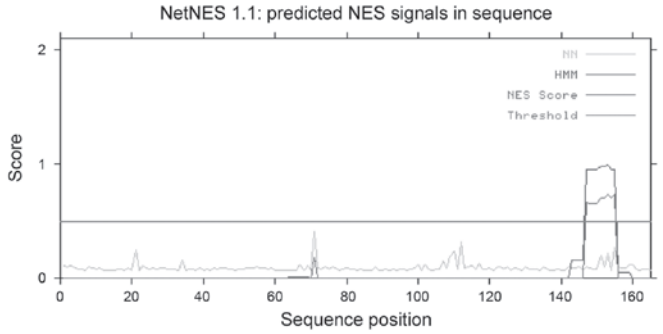

Figure 9. NetNES result.

[Theoretical pI: 8.44 / Mw (average mass): 18750.93 / Mw (monoisotopic mass): 18739.35]

mass	position	#MC	modifications	peptide sequence
5281.2709	83-132	0		NPDYEESGESSSSGGSELEP SGHQLFCLEYEADSGEVTSV IVYQGDDPGK
1886.8683	1-18	0		MEAGPHPRPGHCCKPGGR
1475.7324	19-30	0		LDMNHGFVHHIR
1320.6728	137-148	0		VSAHTPLDPPMR
1228.7048	64-73	0		KPDLQVYLPR
1032.5697	55-63	0		HTPAPTRPR
830.4618	155-161	0		IQEEIAK
781.3951	76-82	0		DVSAHPR
655.2569	37-41	0		DDYDK
601.3416	32-36	0		NQIAR

Figure 10. PeptideMass.

1 - 8	895.02	7.65	MEAGPHPR
1 - 14	1520.78	8.24	MEAGPHPRPGHCCK
1 - 18	1888.19	8.98	MEAGPHPRPGHCCKPGGR
9 - 14	644.79	8.29	PGHCCK
9 - 18	1012.19	9.42	PGHCCKPGGR
9 - 30	2469.86	9.42	PGHCCKPGGRLDMNHGFVHHIR
15 - 30	1844.09	11.38	PGGRLDMNHGFVHHIR
15 - 31	2000.28	12.21	PGGRLDMNHGFVHHIRR
19 - 30	1476.69	8.09	LDMNHGFVHHIR
19 - 31	1632.87	10.94	LDMNHGFVHHIRR
19 - 36	2215.53	12.20	LDMNHGFVHHIRRNQIAR
31 - 36	757.86	12.50	RNQIAR
31 - 41	1394.47	6.67	RNQIARDDYDK
32 - 41	1238.28	4.21	NQIARDDYDK
32 - 42	1366.46	6.57	NQIARDDYDKK
37 - 42	783.80	4.21	DDYDKK
37 - 44	1011.11	6.93	DDYDKKVK
42 - 48	772.95	10.81	KVKQAAK
43 - 48	644.78	10.60	VKQAAK
43 - 50	902.07	10.32	VKQAAKEK
45 - 50	674.76	9.62	QAAKEK
45 - 52	930.08	10.52	QAAKEKVR
53 - 61	1092.23	12.80	RRHTPAPTR
54 - 61	936.05	12.50	RHTPAPTR
54 - 63	1189.35	12.80	RHTPAPTRPR
55 - 61	779.86	10.85	HTPAPTR
55 - 63	1033.16	12.50	HTPAPTRPR
55 - 64	1161.34	12.51	HTPAPTRPRK
62 - 73	1482.75	10.70	PRKPDLQVYLPR
64 - 73	1229.45	9.45	KPDLQVYLPR
64 - 75	1522.78	10.33	KPDLQVYLPRHR
65 - 73	1101.28	6.94	PDLQVYLPR
65 - 75	1394.60	10.34	PDLQVYLPRHR
65 - 82	2157.42	10.33	PDLQVYLPRHRDVSAHPR
74 - 82	1075.16	10.70	HRDVSAHPR
76 - 82	781.84	7.90	DVSAHPR
133 - 148	1765.02	7.82	VSEKVSAHTPLDPPMR
133 - 152	2206.55	7.80	VSEKVSAHTPLDPPMREALK
137 - 148	1321.53	7.85	VSAHTPLDPPMR
137 - 152	1763.05	7.82	VSAHTPLDPPMREALK
137 - 154	2032.40	10.09	VSAHTPLDPPMREALKLR
149 - 154	729.89	10.10	EALKLR
149 - 161	1541.81	7.18	EALKLRIQEEIAK
153 - 161	1100.29	7.12	LRIQEEIAK
153 - 162	1256.47	10.09	LRIQEEIAKR
155 - 161	830.94	4.31	IQEEIAK
155 - 162	987.13	7.16	IQEEIAKR

Figure 11. Mass spectrometry result.

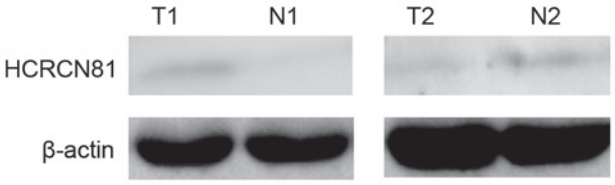

Figure 12. Western blot analysis. T1, protein sample from tumor tissues of a 74-year-old female patient with rectal tubular and villous carcinoma complicated with moderate epithelial hyperplasia. N1, protein sample from normal tissues 5 cm from the tumor mass. T2, protein sample from tumor tissues of a 76-year-old male patient with rectal adenocarcinoma. N2, protein sample from normal tissues 5 cm away from the tumor mass.

Mass spectrometric analysis revealed that the protein molecule contained 165 amino acids, had lost a histidine in the terminal region, had a monoisotopic MW of 18.6033 and a pI of 8.43. The sequences are shown in Fig. 11, which covered 81.3% of the entire protein molecule. Taking the remaining the peptide sequences into consideration, results of mass spectrometry were considered to be accurate.

Western blot analysis demonstrated a clear band at the position of 18 kDa (Fig. 12).

Discussion

Following completion of the Human Genome Project and current progression of the post-Human Genome Project, a large amount of genomic information is available at present. To improve understanding of the significant amount of the notable increase of biological information, bioinformatics has become a useful tool for biological and biomedical studies. In the early stages of the Human Genome Project, small clones were prioritized for full-length sequencing (12). A number of software and network tools have been significant in cloning novel genes. In particular, a large amount of information on expressed sequence tags (ESTs) has led to marked changes in the methods used for identifying and cloning novel genes. A series of gene expression analyses (13) and large-scale sequencing studies of ORF tags have made a significant

contribution to the definition of the transcriptome and the improvement of genome annotation (14).

In the present study, a number of network tools and software sets were used to analyze the target sequence, which was beneficial in studying the function of the HCRCN81 protein. More specifically, the full-length cDNA sequence from the NCBI database was used, BLAST was used to search the ESTs and DNAstar was employed for *in silico* cloning. An overlap of two EST alkali bases of >40 bp, with a shared similarity of 95% in the overlapping region, confirmed an accurate match in sequences (15). Following this, the EST was extended for as long as possible and the matching sequences were assembled together forming a new EST for BLAST analysis to identify more matching sequences. This approach was repeated until no more matching sequences were found. Following this, the obtained full-length cDNA sequence was analyzed using bioinformatic tools and databases from the internet (16) and a novel protein, HCRCN81, involved in human CRC, was identified. For this identification, the first step was to translate the cDNA sequence into an amino acid sequence. Initially, numerous initiation codons were identified in NM_001013649. Following this, ORF was used for analysis and it was noted that the majority of these initiation codons resulted in sequences that were too short for a protein molecule. Therefore, the longest peptide sequence was determined as the protein molecule. This result was validated by uniGene in NCBI. The full-length amino acid sequence was then obtained and the physical and chemical characteristics of the protein molecule were analyzed using multiple tools. In particular, ProtParam and ProtScale provided by ExPASy were used to determine the MW and theoretical pI. Additional software sets and tools were used to determine the hydrophobic region, phosphorylation sites and *O*- and *N*-linkage glycosylation sites within the sequence. Following this, the protein structure was predicted. Jpred and PredictProtein were used to analyze the secondary structure. Results of the two approaches were consistent. To predict the tertiary structure of the target protein, the sequence was analyzed using SWISS-MODEL and CBS. The SWISS-MODEL did not yield a result, however, CBS analysis revealed a partial structure of the protein between 108-166 amino acids.

In addition, the function of this protein was investigated. Since NLS and NES were predicted to be present in this protein, it was speculated that the protein may be involved in nucleic function. The outer capsid protein σ3 is capable of stimulating translation by blocking the activation of the dsRNA-dependent protein kinase, EIF2AK2/PKR (17). The function of methenyltetrahydromethanopterin cyclohydrolase is to catalyze the reversible interconversion from 5-formyl-H(4)MPT to methenyl-H(4)MPT(+), by performing the following catalytic reaction: 5,10-methenyl-5,6,7,8-tetrahydromethanopterin + H_2O = 5-formyl-5,6,7,8-tetrahydromethanopterin. In addition, casein kinase II activation has been reported as a downstream event of Wnt signaling activation (18) and tyrosine kinases have been found to transport into the nuclear regions where gene expression may be modified (19). Considering the presence of numerous potential phosphorylation sites, particularly kinase phosphorylation sites in the EF-hand domain, it was hypothesized that, following synthesis, this target protein may be transported into the nucleus, due to the presence of an NLS, which may be phosphorylated, leading to the exposure of its functional regions. It may then bind to Ca^{2+} through the EF-hand domain (10), exposing the NES region, which, in turn, may lead to its transportation out of the nucleus. Consistent with this hypothesis, it has been reported that the EF-hand is directly associated with chronic inflammatory disorders and cancer (15). However, the function of the UPF0651 family remains unclear. A previous study observed downregulation of the mRNA of this gene in human CRC (5). Combined, these observations indicate that this novel protein is potentially key to the development of human CRC.

Mass spectrometry was used to confirm the hypothesis. PeptideMass was used to predict the small peptide sequences following trypsin treatment. By comparing the prediction and the mass spectrometry results, it was confirmed that the prediction of the amino acid sequence of the protein HCRCN81 was accurate.

Western blot analysis, using antibody serum specific for HCRCN81, revealed that HCRCN81 was expressed in tumor and adjacent normal tissues. Previous studies (4,5) have revealed that mRNA expression of NM_001013649 is downregulated in human CRC tissue. Among 30 human CRC tissue samples, 5 revealed upregulated NM_001013649 mRNA expression, whereas 25 demonstrated downregulated NM_001013649 mRNA expression, accounting for 83% of all tested samples. This observation indicated the potential involvement of NM_001013649 in CRC pathogenesis. In addition, NM_001013649 downregulation was detected in 91% of the moderately differentiated samples (21/23) but only in 50% of the poorly differentiated tissue samples (3/6) and the difference was considered to be statistically significant (Fisher's exact probability test, $P<0.05$). This observation reveals a possible correlation between NM_001013649.3 transcriptional expression and CRC tumor stage.

In the present study, using bioinformatic tools, the structure of a novel protein, HCRCN81, translated from NM_001013649.3 mRNA, was analyzed. Using western blot analysis, protein expression of HCRCN81 in cell lines and colorectal tissue samples was detected, with a MW of ~18 kDa, consistent with the hypothesis. In addition, potential NLS and NES from the protein sequence of HCRCN81 and the EF-hand domain were predicted. This observation was in accordance with the hypothesis that HCRCN81 may function as a cell cycle regulator in the nucleus, which, in turn, may explain its possible role in CRC pathogenesis. Mass spectrometry was used to determine the amino acid sequence of the protein, the result of which was consistent with the prediction using bioinformatic tools. The bioinformatic analysis of the present study also indicated that HCRCN81 is a member of the UPF0561 family. The protein function of the UPF0561 family has not been well characterized. The current study indicates that proteins of the UPF0561 family may have a similar function to HCRCN81 in cell cycle regulation and additional studies on this family must be performed.

Acknowledgements

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