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EXPRESSION PATTERNS OF THE HUMAN RIBOSOMAL PROTEIN GENES *eL14* AND *uS19* IN COLON CANCER IS DEPENDENT ON THE TYPE AND STAGE OF THE CANCER CELL

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ABSTRACT

Although the association of some ribosomal protein genes with colorectal cancer is widely known, the detailed mechanisms and complete list of associated genes is lacking. More importantly, the behaviours of these genes in different types and stages of the cancer are poorly understood. Herein we report the study of two ribosomal protein genes in cell lines derived from different sites and stages of colon cancer. Specifically, we analysed the expression pattern of *eL14* and *uS19* in HCT116 and SW480 cell lines. These two genes, although associated with a wide variety of cancer types, are poorly or have not been studied in colorectal cancer. Semi-quantitative reverse transcription – polymerase chain reaction (RT-PCR) approach was used, together with Students' *t*-test validation. We found a significantly ($p < 0.05$) differential *eL14* and *uS19* expression patterns between HCT116 and SW480 cell lines. Our findings suggest that *eL14* and *uS19* have higher activity in a poorly differentiated cell line derived from advanced (metastatic) stage (Duke's Stage D) colorectal carcinoma tissues compared to the moderately differentiated cell line derived from a mid-stage (Duke's Stage B) colorectal adenocarcinoma tumour. This will have important implications for both ribosomal protein genes as type and stage specific biomarkers for colon cancer.

Key words: Ribosomal protein genes, colon cancer, transcript level, *eL14*, *uS19*

INTRODUCTION

Differential expression of ribosomal protein (RP) genes is widely reported in a variety of cancer types. Over the years, there is increasing evidence of their association with colorectal cancer (Pogue-Geile *et al*., 1991; Kondoh *et al*., 1992; Wang *et al*., 2000; Shenoy *et al*., 2012; Yu *et al.*, 2019). Patterns of their expression also varies with different stages of malignancy (Lai & Xu, 2007), thus substantiating their involvement in the carcinogenesis of colon cancer. While some RP genes have elevated expression in colorectal cancer (CRC), several others are down-regulated, particularly in metastatic CRC (Lai & Xu, 2007). These RP genes are suspected to have specific roles in tumour progression and cancer metastasis, and cellular differentiation in CRC (Gou *et al*., 2010). However, not enough is known about the whole list of RP genes that is associated with CRC. Current literature lists at least 80 members of the RP gene family in existence (Ban *et al*., 2014). Exactly how many and which of these are linked to colorectal cancer is yet to be clarified. At what stages of the cancer are they most active also remains to be fully elucidated.

We sought to study the ribosomal protein gene L14 (*eL14*) in the context of colorectal carcinoma because its link to the different stages of malignancy of the cancer has never been properly studied. *eL14* is located at a chromosomal position 3p21.3 (Huang *et al*., 2006) – a region where the loss of heterozygosity (LOH) is associated with several types of cancer, and possibly have tumour suppressor genes (Liu *et al*., 2003). *eL14* has been found to be differentially expressed in lung, oral, (Shriver *et al*., 1998), oesophageal (Huang *et al*., 2006), and nasopharyngeal (Sim *et al*., 2018) cancers. More importantly, it is strongly correlated to microsatellite instability in CRC cases (Yu *et al.*, 2019). In the case of RP gene S15 (*uS19*), despite

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an association with CRC (Lai & Xu, 2007), nothing is known about its activity in the different types and stages of the cancer. Besides colon cancer, *uS19* is highly expressed in esophageal cancer and neuroblastomas (Walter *et al*., 1996), hepatocellular carcinoma (Yoon *et al*., 2006), and nasopharyngeal carcinoma (Fang *et al*., 2008; Sim *et al*., 2018). The uS19 protein is differentially expressed in human gastric carcinoma cells (Mao *et al.*, 2016). Its mutations have been implicated in chronic lymphocytic leukemia (Ljungstrom *et al.*, 2016; Sulima *et al.*, 2017; Bretones *et al.*, 2018; Ljungstrom & Rosenquist, 2018). It is possibly associated with the modulation of the p53 tumour suppressor function via the MDM-2-p53 pathway (Daftuar *et al*., 2013). This has made it an interesting RP genes to be included in our study.

The two CRC cell lines (HCT116 and SW480) employed in our study were derived from different cell types and stages of the cancer. This suited our purpose of investigating the activities of *eL14* and *uS19* in different types and stages of CRC. HCT116 is a growth factor independent epithelial cell line (Rajput *et al*., 2008) derived from the Duke's Stage D (metastatic) cells of the ascending colon of a 48 years old male diagnosed with colorectal carcinoma (Niu *et al*., 2012). It is of tumour Grade IV and comprising poorly differentiated cells (Niu *et al*., 2012) that are largely cancer stem cells (Yeung *et al*., 2010). In contrast to HCT116, the SW480 cell line originated from tumour tissues of the descending colon a 50 years old male with Duke's Stage B (non-metastatic) colorectal adenocarcinoma (Flatmark *et al*., 2004). It is of tumour Grade III, consisting of moderately differentiated epithelial-like cells that have glandular characteristics (Romano *et al*., 2009; Niu *et al*., 2012).

This paper reports the expression behaviours at the transcript level of *eL14* and *uS19* in HCT116 and SW480 cell lines with the aim of identifying and/or establishing association of these RP genes in CRC, and to unravel their activities in different types and stages of the cancer.

MATERIALS AND METHODS

Cell lines and culture

The colorectal cell lines used for this study are HCT116 and SW480. Both cell lines were purchased from American Type Cell Culture (ATCC). HCT116 was derived from cells of the ascending colon of a 48 years old man with CRC. SW480 cell lines originated from the descending colon of a 50 years old man with colorectal adenocarcinoma. HCT116 were cultured in McCoy 5A media supplemented with 10% Foetal Bovine Serum (FBS). Culture conditions were at 37°C with 5% CO_2, and 5% humidity. SW480 were cultured in L15 media supplemented with 10% FBS. Culture conditions were at 37°C with 5% humidity. Cells were harvested for total RNA extraction when they reached 70–80% growth confluence.

Total RNA extraction

Total RNA was extracted using the TRIzol method (Chomczynski & Sacchi, 1987). Prior to the addition of TRIzol reagent (Invitrogen™, USA), cells were washed in cold phosphate buffer saline (PBS) solution. Suspension mixture in T25 culture flasks was transferred to 1.5 ml sterile tubes, mixed by gentle pipetting, and left at room temperature (RT) for 5 minutes. Chloroform (1/5 vol.) was then added, and the lysate was briefing vortexed and left at RT for 2 minutes. This was centrifuged at 12,000 rpm for 15 minutes at 4°C. Then, the aqueous phase was transferred into a new 1.5 ml tube, added with 0.5 ml isopropanol and left at RT for 10 minutes. Centrifugation at 10,000 rpm and 4°C for 10 minutes followed. Then the supernatant was carefully removed leaving the RNA pellet, which was washed with 1 mL of 75% ethanol, and air dried for 5 minutes. It was then dissolved in nuclease-free water stored at -80°C until use. Quantity and quality assessments were via agarose gel electrophoresis and UV spectrophotometry assays.

Reverse Transcription – Polymerase Chain Reaction (RT-PCR) assay

Conversion of RNA to cDNA was carried using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) (Promega, USA). RNA samples were mixed with random primers in a sterile 1.5 mL tube, and incubated at 70°C for 5 minutes. Following that, the mixture was immediately cooled on ice for 1 minutes, vortexed, and added with 1X M- MLV reaction buffer, 1 mM dNTPs, Recombinant RNAsin® Ribonuclease Inhibitor, and 1U of M- MLV RT. This was incubated at 37°C for 60 minutes, and then at 70°C for 15 minutes. Enzymatic amplification of eL14 and uS19 via PCR was according to the following conditions and parameters. Each reaction comprised 1X Green GoTaq® Flexi Buffer, 1mM MgCl2, 0.2mM dNTP Mix, 0.4 µM forward primer, 0.4 µM reverse primer, and 0.6U GoTaq® Polymerase, and 0.5 µg template DNA. Negative control contained no template DNA in the reaction mix. PCR test using *GAPDH* was used as a control for monitoring comparable amount use of template DNA. The PCR primers used in our study include the forward and reverse primers of *eL14* (5'-ATGGTGTTCAGGCG CTTC G-3'; 5'-CCACTGTCAAGCTGCCTCC-3'), *uS19* (5'-GAAGACGCACCTGCGGGACA-3'; 5'-GTGGCCGATCATCTCGGGCTTG-3'), and *GAPDH* (5'-AGATCATCAGCAATGCCTC-3'; 5'-TACC

AGGACATGAGCTTGAC-3'). Expected amplicon sizes for the primer pairs are 760 bp (*eL14*), 115 bp (*uS19*), and 508 bp (*GAPDH*). The thermal cycling parameters of PCR assay for each gene were as follows: For *eL14*, 30 cycles of denaturation at 95°C for 0.5 minutes, annealing at 57°C for 0.5 minutes, and extension at 72°C for 1 minutes; for *uS19*, eL14, 30 cycles of denaturation at 95°C for 0.5 minutes, annealing at 57.6°C for 0.5 minutes, and extension at 72°C for 1 minutes; and for GAPDH, 30 cycles of denaturation at 95°C for 0.5 minutes, annealing at 50.7°C for 0.5 minutes, and extension at 72°C for 1 minutes. Assessment of PCR assays was via agarose gel electrophoresis (AGE), and band intensities of amplified products were documented and quantified using ImageQuant Imager and associated software (GE Healthcare Life Sciences, USA). Amplicons were also verified by sequence analysis.

DNA analysis

Expression patterns of each gene were measured based on band intensities of RT-PCR products observed on AGE assay. Biological replicates (duplicates) were implemented in this study. Data normalization was done by comparing values of RP genes with the housekeeping gene, *GAPDH*. Differential expression of RP genes between the two cell lines was evaluated using paired Student's *t* – test, with significance taken at p-value < 0.05.

RESULTS AND DISCUSSION

***eL14* expression pattern in colon cancer cell lines**

The *eL14* expression as detected in the colorectal carcinoma cell line (HCT116) but not in the colorectal carcinoma cell line (SW480). Electrophoretic results of this observation (Figure 1(A)) were substantiated by band intensity assessment (Table 1), and illustrated in a bar chart (Figure 1(B)). Sequence analysis verified the authenticity of the amplicon, as bone fide PCR-amplified products of the *eL14* gene (85% identical with reference data in GenBank, Fig. 3(A)). Amplicons of the housekeeping gene (as internal control), *GAPDH* was also sequence verified (96% identical with reference data in GenBank, Fig. 3(C)). It is clear that the expressed transcript level between the two cell lines is significantly different even without inferential statistical evaluation. Despite the fact that high band intensity value (mean normalized value of 36150.90 ± 967.19) was evident in HCT116 cell lines, no detectable expression (band intensity value of 0.00) was observed in SW480 cells (Figure 1, Table 1).

The obvious expression deficiency in SW480 is not an unexpected result, albeit proven for the first time in a colon cancer model in this study. Early and circumstantial evidence of *eL14* inactivation in cancer was from observation of frequent loss of heterozygosity at chromosomal region 3p21.3 in squamous cell carcinoma of the head and neck (SCCHN) (Shriver *et al*., 1998). The *eL14* gene resides within this region. The cause of *eL14* loss and/or inactivation in cancers is still unknown. Interestingly, our study unveiled expression of *eL14* in HCT116 cell line. This provides evidence that *eL14* is not necessary inactivated or down-regulated in cancer cells, thus adding to the complexity of its involvement in cancer, particularly colon cancer. This difference in expression could be partly attributed by disparity in cell types between HCT116 and SW480. Both originated from different sites of the colorectal organ (HCT116 and SW480 from ascending and descending colon respectively), composed of dissimilar degree of cellular differentiation (HCT116 is poorly differentiated whereas SW480 is moderately differentiated epithelial-like form with glandular characteristics), and were from different stages of malignancy (HCT116 is at Duke's Stage D metastatic status while SW480 is at Duke's Stage B non-metastatic status). Alteration of gene expression in relation to different stages of CRC is a phenomenon that has been previously reported (Kawada *et al*., 2003; Lai & Xu, 2007). Nevertheless, how these cellular and pathologic differences contribute to the behavioural disparities of *eL14* observed in our study remains to be experimentally explored.

***uS19* expression pattern in colon cancer cell lines**

Our analysis revealed detectable expression of *uS19* in both HCT116 and SW480 cell lines (Figure 2(A), Table 2). Sequence analysis verified the authenticity of the amplicon, as bone fide PCR-amplified products of the *uS19* gene (100% identical with reference data in GenBank, Figure 3(B)). Expression in HCT116 was markedly higher compared to SW480 (Figure 2(B), Table 3). Comparison of mean normalized value of the band intensity for the *uS19* gene amplicons between HCT116 and SW280 revealed of 1.576-fold difference. This difference is significant with p-value of 0.026 following Student's *t*-test analysis.

In contrast to the behavior of *eL14*, expression *uS19* was detected in both HCT116 and SW480. We have also demonstrated that the level of *uS19* was higher in HCT116 than SW480. Unlike *eL14*, association of *uS19* with colon cancer has been previously reported (Lai & Xu, 2007). However, this paper is the first to show evidence of its significantly varied expression between colon cancer cells of different types and stages. *uS19* is highly expressed in poorly differentiated, metastatic CRC cells. Further functional studies on regulatory network of

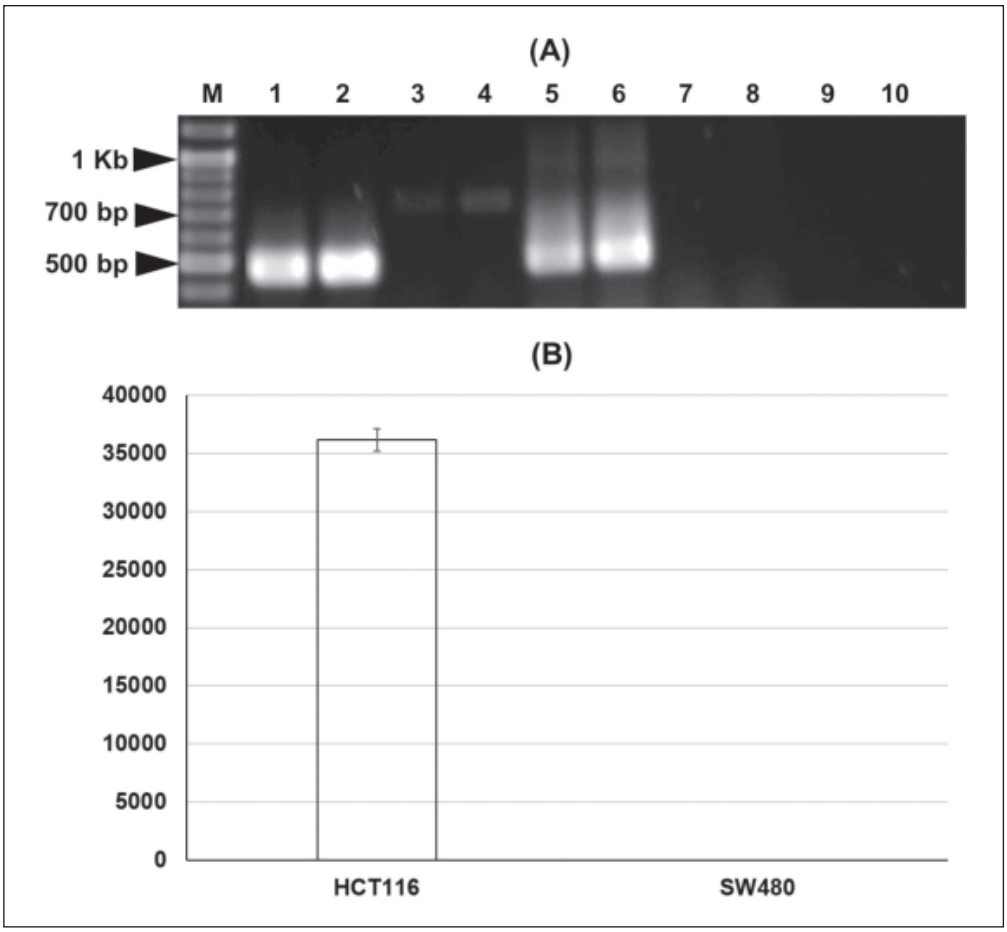

Fig. 1. Expression pattern of *eL14* in HCT116 and SW480 cell lines. (A), Gel electrophoretic results of *eL14* RT-PCR assay (lane M: GeneRuler™ DNA Ladder; lanes 1 and 2: *GAPDH* amplicons in HCT116; lanes 3 and 4: *eL14* amplicons in HCT116; lanes 5 and 6: *GAPDH* amplicons in SW480; lanes 7 and 8: *eL14* amplicons in SW480; and lanes 9 and 10 are negative controls of *eL14* test for HCT116 and SW480 respectively). (B), Column chart of mean band intensity value with standard deviation error bar of *eL14* amplicons in both colon cancer cell lines. The y-axis represents band intensity values, and the x-axis is the type of cell lines.

Table 1. Band intensity value of *eL14* and *GAPDH* amplicons in HCT116 and SW480 cell lines

Cell lines	Replicate test	Band intensity value				Standard Deviation
		eL14	*GAPDH*	Normalized *eL14*	Mean normalized *eL14*	
HCT116	1	36321.33	165835.28	35474.77	36150.90	956.19
	2	38293.54	163290.25	36827.03		
SW480	1	0.00	173787.09	0.00	0.00	0.00
	2	0.00	176258.23	0.00		

uS19 in CRC carcinogenesis would be required to unravel the molecular rationale underlying this observation.

Our findings on *eL14* and *uS19* suggest a marked or elevated expression of these genes in metastatic rather non-metastatic colon cancer cells. Although differential expression of up to 18 RPs in tumour cell lines of the same origin but different metastatic abilities has been reported (Kreunin *et al*., 2007), our study is the first to demonstrate this for *eL14* and *uS19* in colorectal malignancy. This implies biomarker of prognostic potential for these two RP genes in carcinogenesis of the colorectum. Nonetheless, further evaluation of their protein

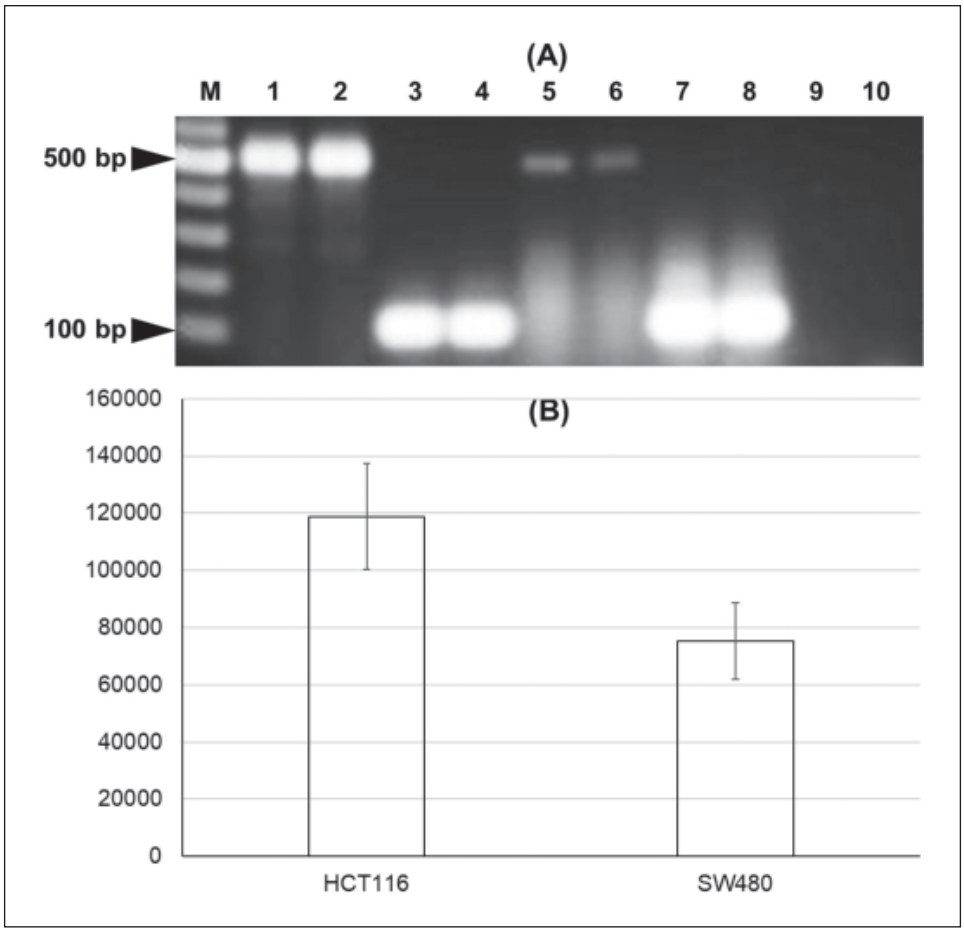

Fig. 2. Expression pattern of *uS19* in HCT116 and SW480 cell lines. (A), Gel electrophoretic results of *uS19* RT-PCR assay (lane M: GeneRuler^{TM} DNA Ladder; lanes 1 and 2: *GAPDH* amplicons in SW480; lanes 3 and 4: *uS19* amplicons in SW480; lanes 5 and 6: *GAPDH* amplicons in HCT116; lanes 7 and 8: *uS19* amplicons in HCT116; and lanes 9 and 10 are negative controls of *uS19* test for HCT116 and SW480 respectively). (B), Column chart of mean band intensity value with standard deviation error bar of *uS19* amplicons in both colon cancer cell lines. The y-axis represents band intensity values, and the x-axis is the types of cell line.

Table 2. Band intensity value of *uS19* and *GAPDH* amplicons in HCT116 and SW480 cell lines

Cell lines	Replicate test	Band intensity value				Standard Deviation
		uS19	*GAPDH*	Normalized *uS19*	Mean normalized *uS19*	
HCT116	1	120113.63	19152.49	105669.2522	118773.54	18532.23
	2	144055.89	19930.11	131877.8364		
SW480	1	62214.00	23019.64	65783.61721	75267.80	13412.67
	2	73863.32	24979.86	84751.99347		

Table 3. Student's *t*-test analysis of uS19 expression level between HCT116 and SW480

Cell lines	Mean (band intensity)	Standard deviation	*t*-value	*p*-value
HCT116	118773.54	18532.23	6.314	0.026

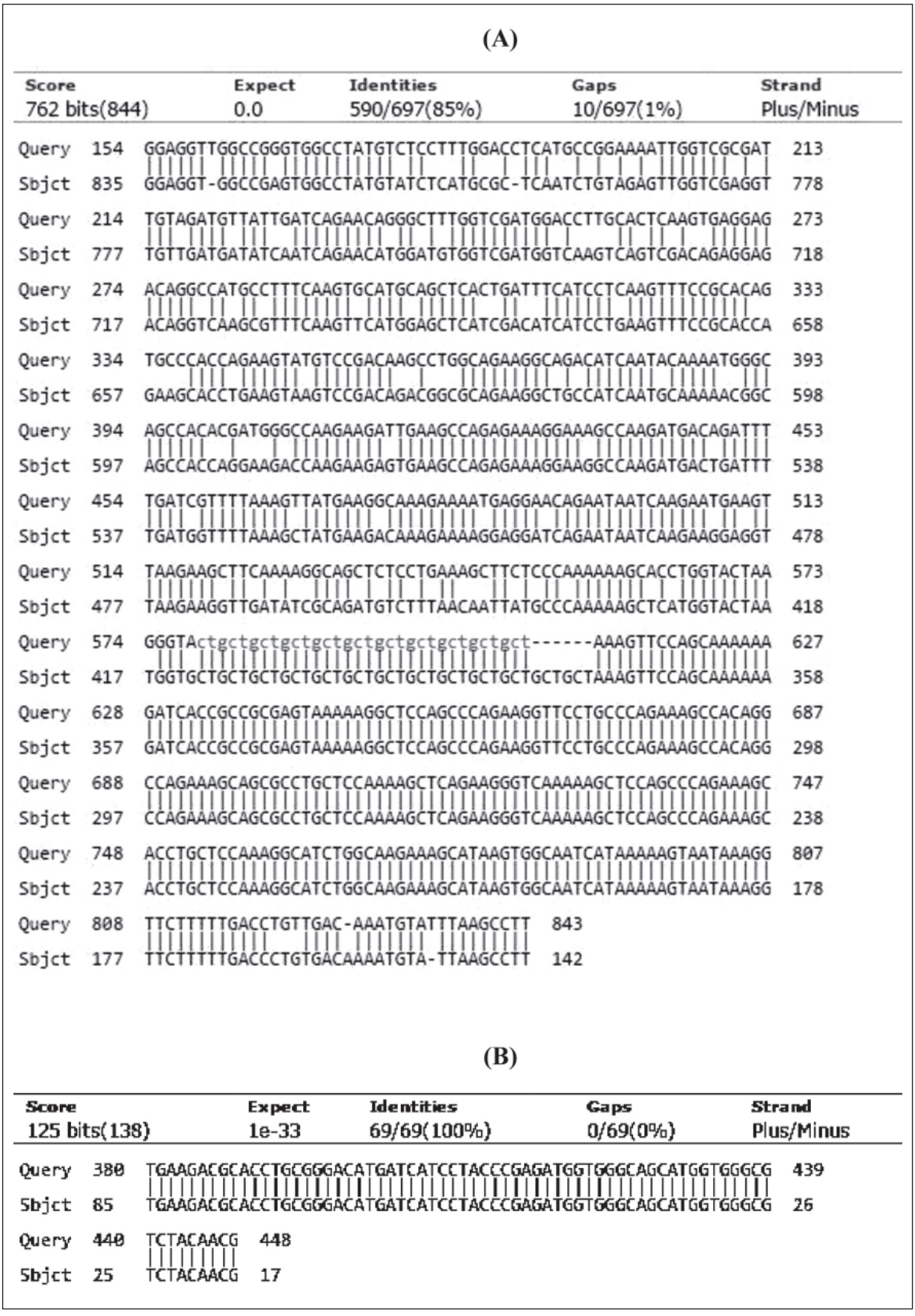

Fig. 3. Sequence verification via comparison of gene-of-interest sequence with reference data in GenBank: (A), *eL14*; (B), *uS19*; and (C), *GAPDH*.

Figure 3 continued...

(C)

Score	Expect	Identities	Gaps	Strand
812 bits(900)	0.0	453/455(99%)	0/455(0%)	Plus/Plus

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Query  661   GTCATCCATGACAACTTTGGTATCGTGGAAGGACTCATGACCACAGTCCATGCCATCACT  720
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Sbjct  19    GTCATCCATGACAACTTTGGTATCGTGGAAGGACTCATGACCACAGTCCATGCCATCACT  78

Query  721   GCCACCCAGAAGACTGTGGATGGCCCCTCCGGGAAACTGTGGCGTGATGGCCGCGGGGCT  780
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Sbjct  79    GCCACCCAGAAGACTGTGGATGGCCCCTCCGGGAAACTGTGGCGTGATGGCCGCGGGGCT  138

Query  781   CTCCAGAACATCATCCCTGCCTCTACTGGCGCTGCCAAGGCTGTGGGCAAGGTCATCCCT  840
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Sbjct  139   CTCCAGAACATCATCCCTGCCTCTACTGGCGCTGCCAAGGCTGTGGGCAAGGTCATCCCT  198

Query  841   GAGCTGAACGGGAAGCTCACTGGCATGGCCTTCCGTGTCCCCACTGCCAACGTGTCAGTG  900
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Sbjct  199   GAGCTGAACGGGAAGCTCACTGGCATGGCCTTCCGTGTCCCCACTGCCAACGTGTCAGTG  258

Query  901   GTGGACCTGACCTGCCGTCTAGAAAAACCTGCCAAATATGATGACATCAAGAAGGTGGTG  960
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Sbjct  259   GTGGACCTGACCTGCCGTCTAGAAAAACCTGCCAAATATGATGACATCAAGAAGGTGGTG  318

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Sbjct  379   TCCTCTGACTTCAACAGCGACACCCACTCCTCCACCTTCGACGCTGGGGCTGGCATTGCC  438

Query  1081  CTCAACGACCACTTTGTCAAGCTCATTTCCTGGTA  1115
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Sbjct  439   CTCAACGACCACTTTGTCAAGCTCATGTCCTGGTA  473
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levels in the tested cell lines, including non-malignant cell lines, is still required to verify our suspicion.

CONCLUSION

The RP genes of *eL14* and *uS19* are highly expressed in the poorly differentiated metastatic cell line, HCT116 that originated from the ascending colon of colorectal carcinoma tissue. In the moderately differentiated epithelial-like (with glandular characteristics) non-metastatic cell line, SW480 that was derived from the descending colon of colorectal adenocarcinoma tissue, *eL14* was not detected and *uS19* was expressed but at a significantly lower level compared to HCT116. Both *eL14* and *uS19* have potential as biomarkers to distinguish between different types and stages of colon cancer.

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