Heliyon 10 (2024) e27475

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Updating mRNA variants of the human RSK4 gene and their expression in different stressed situations

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ARTICLE INFO

Keywords:
RSK4
Alternative splicing
Protein isoform
Polycistron

ABSTRACT

We determined RNA spectrum of the human RSK4 (hRSK4) gene (also called RPS6KA6) and identified 29 novel mRNA variants derived from alternative splicing, which, plus the NCBI-documented ones and the five we reported previously, totaled 50 hRSK4 RNAs that, by our bioinformatics analyses, encode 35 hRSK4 protein isoforms of 35–762 amino acids. Many of the mRNAs are bicistronic or tricistronic for hRSK4. The NCBI-normalized NM_014496.5 and the protein it encodes are designated herein as the Wt-1 mRNA and protein, respectively, whereas the NM_001330512.1 and the long protein it encodes are designated as the Wt-2 mRNA and protein, respectively. Many of the mRNA variants responded differently to different situations of stress, including serum starvation, a febrile temperature, treatment with ethanol or ethanol-extracted clove buds (an herbal medicine), whereas the same stressed situation often caused quite different alterations among different mRNA variants in different cell lines. Mosifloxacin, an antibiotics and also a functional inhibitor of hRSK4, could inhibit the expression of certain hRSK4 mRNA variants. The hRSK4 gene likely uses alternative splicing as a handy tool to adapt to different stressed situations, and the mRNA and protein multiplicities may partly explain the incongruous literature on its expression and comports.

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https://doi.org/10.1016/j.heliyon.2024.e27475
Received 20 January 2024; Received in revised form 11 February 2024; Accepted 29 February 2024
Available online 8 March 2024

1. Introduction

Almost all human genes employ multiple mechanisms to produce multiple variants of mRNA and/or long noncoding RNA, besides assorted small regulatory RNAs, to diversify the genes' functions [1–3]. For example, many human genes use different initiation sites or termination sites of transcription to produce multiple pre-RNA transcripts, and in most cases the transcripts undergo alternative splicing (AS) [4–6]. It has been estimated that the roughly 20,000 protein-coding genes in the human genome produce about 64,000 [7,8] or 86,000 [9,10] coding transcripts, which together constitute 1.91% of the 3.1 gigabases of the genome [11–13]. In addition, it has been estimated that one in five human genes still have unresolved coding status [14]. These data suggest that most human genes should produce multiple protein isoforms, roughly three or four proteins per gene. However, some RNA pundits consider that a significant number of AS products may be noise or be precarious [9,15–18] and that alternative initiation of transcription is largely nonadaptive [19]. Results of some proteomics studies suggest that each gene has a principal protein form [7,9,15], which in our opinion should be the wild type (Wt). Indeed, most published Western blotting (WB) data show only one band on the WB membrane, insinuating that there only is one protein form expressed. Therefore, there is a clear dissonance between the RNA profile and the protein profile in a global view, which in our opinion has several possible explanations. One is that routine LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry) proteomic approach involving a bottom-up strategy may not be able to detect short protein isoforms of the same gene [20–23]. Another reason is that a particular cell or tissue type in a particular physiological or pathological situation may only need to express one protein form, theoretically [3]. A third possible reason, which we have discussed previously [3,21–24], pertains to researchers' conduction of WB or related techniques: many researchers prefer the antibodies that recognize only the protein isoform of interest while considering the antibodies that produce additional bands on the WB membranes "not specific enough" and abandoning them. Actually, there often are additional bands on the WB membrane. However, a common but unmentioned practice is to assume them as artifacts and thus to cut them off, leaving only the band of interest to be presented in publication. This kind of practice prompts most commercial antibody suppliers to select and commercialize the antibodies that recognize only one of the multiple isoforms, usually the Wt, in order to avoid being blamed for selling "not specific enough antibodies".

Splicing of a pre-RNA transcript occurs co-transcriptionally, i.e., almost simultaneously with its transcription from DNA template [5,25]. However, as a major mechanism for RNA diversification, AS may occur later to a spliced RNA, especially when the initial splicing is a "heterosplicing", viz. a splicing of a transcript from a recombinant DNA [5,26–29]. A possible reason is that the recombinant DNA is somewhat foreign to the cell, and part(s) of this somewhat alien sequence are recognized by the cell as cryptic splice sites [5,27,29]. When the gene to be expressed is heterologous, the chance for the occurrence of heterosplicing is increased because the recombinant DNA is more foreign [5]. Some researchers who lack sufficient experience and knowledge of AS may not deeply meditate on, and thus may not test the possibility of, the occurrence of heterosplicing when designing a recombinant construct of cDNA (complementary DNA) or transgene.

We have long studied the role of the X-linked RSK4 (Ribosomal S6 kinase 4; also called RPS6KA6, ribosomal protein S6 kinase A6) gene in cancer. Initially, we found that this gene in the human and mouse showed a higher expression in breast cancer tissues than in their uninvolved surrounding tissues [30–32], just like many oncogenes. However, breast cancer cell lines that ectopically expressed an hRSK4 cDNA construct manifested tumor-suppressive features both in cell culture and as xenograft tumors in immunodeficient mice [33,34]. This incongruity has continuously appeared in the literature among different cancer types [35], with some studies showing that RSK4 increases its expression or behaves like an oncogene in certain cancer types [36,37] and some others showing the opposite, especially in other cancer types [38,39]. Reiterated, whether RSK4 is an oncogene, a tumor suppressor gene, or a gene having tissue- or cell-specific function remains uncertain. Drawing from our experience in working with transcription and AS of RNA [1,2,25,34,40–45], we wondered whether the incongruity could be attributable partly to the multiplicities of hRSK4 mRNA and protein, although the NCBI (National Center for Biotechnology Information, USA) database listed only one RNA form (NM_014496.2) of the hRSK4 then. In line with this conjecture, our WB analyses using protein samples from multiple breast cancer cell lines and eight different commercial hRSK4 antibodies all detected multiple bands on the WB membranes, varying from about 30 kD to 135 kD, although the hRSK4 protein has always been expected to be about 90 kD [34]. Proteins expressed from an hRSK4 cDNA construct also showed multiple bands on the WB membranes [34]. In addition, our early studies identified a new transcriptional initiation site and several AS-derived mRNA variants of hRSK4 [34].

NCBI has for a few years listed more mRNA variants of hRSK4, ten in total by now, of which two are normalized sequences with their coding proteins differing at the N-terminus. The European database, named Ensembl, also lists four noncoding long RNAs and two mRNAs. However, very few publications on hRSK4 point out the existence of two normalized protein isoforms and connect the RNA and protein multiplicities to the discrepant data in the literature. Actually, there only is one bioinformatics study mentioning the two mRNAs [46]. On the other hand, in our work, and in collaborations with others, we have continuously identified more AS-derived mRNA variants of hRSK4 in different cancer types and in different physiological or pathological situations. In this communication, we report these novel variants along with in-silico analyses of the open reading frames (ORFs) in these unreported variants as well as in the NCBI- and Ensembl-listed sequences, because we surmise that the mRNA and protein multiplicities might partly explain the discrepant data in the literature about the functions of RSK4 in cancer.

2. Materials and methods

2.1. Retrieval of RNA sequences from databases

NCBI updates its database almost daily. On December 1 of 2023, it listed ten hRSK4 mRNA sequences. We downloaded these sequences and the corresponding illustrations of intron and exon organization from "https://www.ncbi.nlm.nih.gov/gene/". NCBI also assembled all 26 exons of the hRSK4 gene (ENSG00000072133) deposited in the Ensembl database into an mRNA and illustrated exon-intron relationship of this assembled mRNA; this illustration was downloaded as well. In addition, NCBI organized many expressed sequence tags (EST) of hRSK4 into six (from "a" to "f") groups and illustrated these groups in a figure, which was downloaded from "https://www.aceview.org".

The European Ensembl has its own database on various genomes, RNA transcripts, and proteins, and annotates them as "ENSG (Ensembl gene), ENST (Ensembl transcript), and ENSE (Ensembl exon)", respectively. We searched for the hRSK4 gene (ENSG00000072133) in the Ensembl database (https://www.ensembl.org) and downloaded the sequences of the hRSK4 RNAs and corresponding exons, along with a relevant table and an illustration.

2.2. Collection and storage of surgically removed oral tissues

During oral surgery performed in the Stomatology Hospital of Guizhou Medical University, pathologically-diagnosed squamous cell carcinoma tissues and some adjacent uninvolved tissues were removed from patients. We collected a small section of these tissues from several patients. The specimens were first stored in liquid nitrogen and later transferred into a −80 °C freezer until use. All participant patients had been informed about the tissue collection, use, and possible publications and had signed a written consent. The sample collection, use, and written consent were approved by the Ethics Committee of the Stomatology Hospital, Guizhou Medical University (No.2021–26). The entire procedure, including the tissue collection and use, was abided by the approved protocol and was in compliance with the principles of the Declaration of Helsinki. None of the patients' names or other personal information were disclosed through the entire study or the present communication.

2.3. Cell lines and cell culture

A panel of human cell lines, including 22RV1, 5637, HEP-3B, MGC-803, CAL-27, HUH-7, SCC-25, HEPG2, HGC-27, OMEC, A2780, SKOV-3, PANC-1, and PANC-28, with their identities provided in the corresponding result description, were initially purchased from the ATCC (American Tissue Culture Collection) by our lab or our collaborators. As a routine, these cells were cultured in flasks at 37 °C or 39 °C for different purposes in a Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) or as indicated. When cell growth reached about 80% or indicated confluence, the cells were detached from the flask by a brief treatment with a DMEM containing 0.25% trypsin-EDTA (Ethylene Diamine Tetraacetic Acid). After removal of the trypsin by washing with a phosphate-buffered saline (PBS), the cells were centrifuged at 5000 rpm for 5 min at room temperature. The cell pellet was used for isolation of total RNA.

2.4. Treatments of cells in culture

In one set of experiments, human gastric cancer cell line HGC-27 and human ovarian cancer cell line A2780 were first cultured as described above. The cells were then harvested and split into three flasks, which were cultured in an DMEM containing 0%, 5%, and 10% FBS, respectively. After three or four additional days of culture the cells were harvested for RNA isolation.

In another set of experiments, human ovarian cancer cell lines A2780 and SKOV-3 were cultured as described above and, when the cell density reached about 50% confluence, received three days of treatment with PBS-diluted mosifloxacin hydrochloride eye drops (Catalog#E2110070; Jiangxi Kelun Pharmaceutical Co., Ltd, Jiangxi China) at a final concentration of 0, 10, 30, 70, or 100 μM. Microscopic observations of the cells did not show obvious cell death during this period. The cells were then photographed and harvested with the procedure described above for RNA isolation.

In a third set of experiments, 1 g of dry clove buds (Catalog#191113; Guizhou Tongji Tang Traditional Chinese Medicine Decoction Pieces Co. Ltd, Guizhou, China), which is a spice in Chinese cuisine and a traditional Chinese herb medicine, was soaked in 5 ml of 95% ethanol at room temperature, as we described before [47]. Ten days later the ethanol was aspirated as the clove infusion and stored at 4 °C for use within a month. Human pancreatic cancer cell lines PANC-1 and PANC-28 were cultured as above described and, when the cell density reached about 50% confluence, were cultured with a DMEM containing 5% FBS and the clove infusion at the indicated concentration. A concentration (v/v) of 0.2% of 95% ethanol was used as the solvent control, which was further controlled using the cells without the ethanol treatment. Three days later, the cells were harvested for RNA isolation.

2.5. CCK8 assay and clonogenic survival assay

PANC-1 and PANC-28 cells were seeded at a density of 1000 cells per well in a 96-well plate and were cultured at 37 °C or 39 °C. Twelve hours later when the cells had attached, the above-described clove infusion was added at the indicated concentration, followed by an additional 72-h culture. The rate of live cells was determined using a commercial Cell Counting Kit-8 (CCK8 kit; Catalog#C0005; Shanghai Taozhu Biotechnology Co., Ltd, Shanghai, China; www.targetmol.cn) and following the reagent's manual as routinely

performed in our lab.

In a separate experiment, PANC-1 and PANC-28 cells were seeded at a density of 1000 cells per well in a 6-well plate. After 12 h the medium was replaced with a DMEM containing 5% FBS and the indicated concentration of the clove infusion, followed by a 72-h culture at 37 °C or 39 °C. Controls included the cells treated with or without the same volume of 95% ethanol used for the highest dose of the clove infusion. After this 72-h treatment, the medium was replaced again with one without the clove or ethanol, and the cells were cultured for an additional five days. The cells were then fixed with methanol-acetic acid at a 3:1 ratio and then stained with 1% crystal violet for 30 min at room temperature as we described previously [47–49]. The colonies in the well were photographed. The experiments were performed more than three times, and representative photos were presented.

2.6. RNA extraction and RT-PCR assay

The tissue samples collected from patients were shredded with a scalpel to tiny pieces and then homogenized in a TRIzol reagent (Catalog#15596026; Thermo Fisher Scientific Co., Ltd, Shanghai, China; www.thermofisher.cn) on ice. The pellets of cultured cells collected from the above-described procedures were also lysed with the TRIzol. Total RNA samples were extracted from the tissue lysates or cell pellets as per manual of the TRIzol reagent. An aliquot of the RNA underwent reverse transcription (RT) in a 10-μl volume of reaction to cDNA using random hexamers and a MMLV Reverse Transcriptase (Catalog#RR036A; Takara Biomedical Technology Co. Ltd, Beijing, China; www.takarabio.com) as per instruction of the reagent. An aliquot of the cDNA was amplified using polymerase chain reaction (PCR) in a 20-μl volume, in which the cDNA template was denatured at 95 °C for 3 min, followed by 30–40 cycles of 95 °C for 20 s, 58–61 °C for 20 s, and 72 °C for 30–60 s (depending on the length of the PCR amplicon). Each PCR performance was ended with a final extension for 3 min at 72 °C. For DNA sequencing, PCR was performed for 40 cycles in order to yield a large amount of amplicon. However, for the semi-quantification purpose, the number of PCR cycles was optimized based on the expression level of the RNA variant in question, so that the reaction was terminated within the linear portion of the amplification. The cDNA loading was normalized using the X-linked gene HRPT1 (hypoxanthine phosphoribosyltransferase 1) as a reference. Commonly, a large volume of RT products was made for semi-quantitation purpose, not only because many PCR performances were needed for different primer pairs but also because the HPRT1 cDNA loading needed to be adjusted several times in PCR assays until the abundance of the amplicon showed in the agarose gel was roughly equal among the samples in the same penal. After the loading was adjusted, the same volume of cDNA sample used for the HPRT1 was used in PCR for amplifying different targets of interest.

An aliquot (in most cases 5 μl) of the PCR products was fractionated in a 1–2% agarose gel containing 0.01% of ExRed, a fluorescent dye (Beijing Zoma Biotechnology Co., Ltd, Beijing, China; Catalog# ZS203-1; www.zomabio.com), to visualize the DNA amplicon. If a PCR amplicon band in the gel showed an unexpected size, it would be excised from the gel. A tiny piece (about 1 mm^3) of the DNA-containing gel slice was used as the template to perform a semi-nested or nested PCR to preliminarily determine whether the amplicon was an unreported RNA variant, as described by us previously [50,51].

Table 1
Primers and their locations used in this study.

PrimerName	Sequence	Location
hRSK4-F6	5′-CAGACTGAAGGGAAACTGTAC-3′	Exon 1
hRSK4-F35	5′-AGATGCTACCATTCGCTCCTC-3′	Exon 1
hRSK4F156	5′-ACTACTCCAGTAGAGGCCAC-3′	Exon 2
hRSK4-F198	5′-GGTGAAATTGATTCACAGTAGG-3′	Exon 2
hRSK4-F298	5′-GTGGAAATTGTTAATCCCTATG-3′	Exon 3
hRSK4-v20F	5′-ATGAGCCAATGGAAGAGGGAG-3′	Exon 4
hRSK4-F450	5′-CTATGAGAAAGCAGATCCTGC-3′	Exon 5
hRSK4-R577	5′-ACACCTTCATTGCATAGAGC-3′	Exon 6
hRSK4-R648	5′-TACTTCCACCAGTATATCCCT-3′	Exon 7
hRSK4-R685	5′-CAGACTGAAGGGAAACTGTAC-3′	Exon 8
hRSK4-R741	5′-GAAAACATCTCCTCCCCTGAG-3′	Exon 8
hRSK4-R830	5′-TGGTGCAGATGATCCAAAGC-3′	Exon 9
hRSK4-F1055	5′-TGCTTACTGGTACTCTGCCAT-3′	Exon 12
hRSK4-R1207	5′-CCAATCTATTTGCTGGATTCC-3′	Exon 13
hRSK4-F1675	5′-CGCTATGGACAACATCCCAA-3′	Exon 18
hRSK4-F2049	5′-GGGATATGATGCTGCTTGTGA-3′	Exon 21
hRSK4-39WTR	5′-TGATGTGGGTCCATATGAAGC-3′	Exon 23
hRSK4-39R	5′-TTGTTCAGCAGTATACCGCTT-3′	Exon 23
hRSK4-R2416	5′-GAAAGGTCTTGTGAGTCAGGG-3′	Exon 24
hRSK4-R2493	5′-CAGGCCAGTTGATGTTCGCT-3′	Exon 24
hRSK4-R2638	5′-CTCACTTCCCCTAAAAATGGG-3′	Exon 24
hRSK4-148R	5′-CAACAGTGCTGTAACACTGGG-3′	Intron 4
hRSK4-159R	5′-CAACAGTGCTGTAACACTGGG-3′	Intron 5
hRSK4-132R	5′-GGTGGCTCACGCCTGTAATCC-3′	Exons 22/23
hHPRT-F103	5′- CTTCCTCCTCCTGAGCAGTC-3′	Exon 1
hHPRT-R663	5′- AACACTTCGTGGGGTCCTTT-3′	Exon 7

Note: The exon/intron number for the hRSK4 and the hHPRT1 is calculated based on the NM_001330512.1 and the NM_000194.3, respectively.

2.7. Strategy for PCR primer design to detect exon exclusion

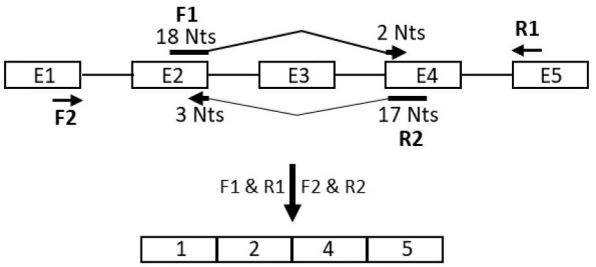
PCR primers used in this study are listed in Table 1. These primers were paired in such a way that the forward (F) and reverse (R) primers were targeted at two different exons for the purpose of determining whether the resulting amplicon is derived from cDNA or from extant genomic DNA. Each F or R primer was named with a number, which in most cases was the distance, viz. how many nucleotides (Nts), from the first Nt of an F primer or the last Nt of an R primer to the first Nt of the NM_001330512.1 sequence, which was used as a reference (unless specified) for the relationships among different RNA variants. With this primer nomenclature, we could calculate the expected size of a PCR amplicon by subtracting the F primer number from the R primer number [20]. If the size of a PCR amplicon differs from the expected size, it is likely an artifact (an off target) or an unreported AS-derived RNA variant.

To specifically amplify an RNA variant with deleted exon(s), either the F or the R primer was divided into a long (usually 17–19 Nts) 5′ part and a short (usually 2–4 Nts) 3' part to target both the recipient exon and the donor exon (Fig. 1), so that annealing of the short part to the donor exon (for the F primer) or the recipient exon (for R primer) would melt during the annealing step of the PCR and thus could not prime an amplification. The 39R primer listed in Table 1 was designed using this strategy. Relative to the variant with an insertion, the one without is the variant with a deletion.

When performing a nested or semi-nested PCR that was an intermediate step in verifying an unknown variant, many primers were designed and used in different combinations, although only the primers used in the final data presentation were reported in the Results section.

2.8. DNA sequencing and in-silico analyses of cDNA and RNA

Once a PCR amplicon band in an agarose gel showed an unexpected size or was likely to be an hRSK4 RNA of interest, we excised the band from the gel and centrifuged the DNA-containing gel slice to separate the DNA from the agarose using a quick method described previously [20,50,51]. The DNA in the supernatant was sent to Diamond Sangon Biotech (Shanghai) Co. Ltd., China (www.sangon.com) for direct sequencing from both ends using the PCR primers. DNA sequences were analyzed using a ClustalW online software (https://www.genome.jp/tools-bin/clustalw), the BLAST (Basic Local Alignment Search Tool) of the NCBI online software (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the BLAT (Basic Local Alignment Tool) of the UCSC (University of California Santa Cruz) online software (http://genome.ucsc.edu/cgi-bin/hgBlat). ORFs of each RNA were identified using a DNAstar software. Alignment of two or more sequences was performed using the ClustalW online software and then edited using BioEditor software or manually in a Microsoft Word document.



acggagcaaag*GATTTGCTTTCCCATATGCTTCATATGGACCCACATCAG*CGGTATACTGCTGAACAAAT
ATTAAAGCACTCATGGATAACTCACAGAGACCAGTTGCCAAATGATCAGCCAAAGAGAAATGATGT
GTCACATGTTGTTAAGgagcaatggttgcaacatactctgccctgactcacaagacctttcaaccagtcctagagcctgt
agctgcttcaagcttagcccagcgacggagcatgaaaaagcgaacatcaactggcct

Fig. 1. Depiction of our primer design strategy for detecting the RNA variants with deletion or insertion of exon(s). For instance, to detect a variant lacking exon 3 (E3), a forward 20-mer (F1) is designed using the last 18 Nts of exon 2 as its long 5′ part and the first 2 Nts of exon 4 as its short 3′ part. RT-PCR using this skewed F1 and a reverse primer (R1) located on exon 5 should amplify only the variant without exon 3. Alternatively, a reverse 20-mer (R2) can be designed to have its first 17 Nts (reverse-complementarily) targeting the first 17 Nts of exon 4 and its last 3 Nts targeting the last 3 Nts of exon 2. RT-PCR using this R2 and a forward primer (F2) located on exon 1 should also amplify only the variant without exon 3. However, this strategy may need modifications, even when the annealing temperature has been raised to a level that decreases the PCR efficiency, because sometimes the last several Nts of a primer may have homologue(s) in one or more regions of the RNA. For instance, our 39R reverse primer (Table 1) designed for specifically amplifying the variant lacking the first 39 Nts (the shaded and italicized sequence) of the penultimate exon (in capital letters, with its flanking exons in lowercase letters) targets the "aagCGGTATACTGCTGAACAA" (underlined) sequence, but its first four nucleotides have several homologues (several underlined AAGC sequences) in the vicinity of the to-be-amplified region. Fortunately, this 4-Nt mis-annealing melts when the annealing temperature is raised to 60 °C.

3. Results

3.1. hRSK4 RNAs in NCBI database

NCBI currently lists two normalized (annotated as NM) mRNA sequences, i.e. NM_014496.5 and NM_001330512.1, and eight predicted (annotated as XM) mRNA sequences of the hRSK4 gene (Fig. 2A). The XM_011530920.3 is the shortest mRNA (having 8154 Nts), processed from the shortest transcript that spans only 106,042 base-pairs (BPs) on the Xq21.1. The XM_017029424.2 is the longest mRNA, having 9379 Nts, but the XM_047441997.1 is processed from the longest transcript that spans 130,154 BPs on the Xq21.1.

The NM_014496.5 has 22 exons and is topped on the NCBI's illustration to indicate that it is the principal or the Wt mRNA (Fig. 2A). The NM_001330512.1 has 24 exons, with its first exon being the last 117 Nts of the first exon of the NM_014496.5, and with its 2nd exon (112 Nts) and 3rd exons (110 Nts) derived from the first intron of the NM_014496.5 (Fig. 2B). The last exon of the NM_014496.5 has 6053 Nts, whereas the last exon of the NM_001330512.1 has 6057 Nts because it has four more Nts (TTTT) at the end (Fig. 2B). The eight predicted mRNAs have 21–25 exons, but all ten mRNAs together involve 26 exons in total. The ten mRNAs differ chiefly at their transcription initiation sites and at the first exon(s) (Fig. 2A) and do not have a poly-A tail.

NCBI assembled all exons and introns of the hRSK4 gene (ESNG00000071233) collected by the Ensembl into an mRNA. We illustrated this assembled mRNA by sketching each exon with its number of Nts (Fig. 2C). This assembled mRNA has two more exons at the upstream of, and far from, the first exon of the NM_014496.5, which makes it the longest mRNA sequence processed from a transcript that spans a much larger region (148,011 BPs) of the Xq21.1 (Fig. 2C).

NCBI also contains many ESTs of hRSK4 and has categorized these sequences into the a, b, c, d, e, and f groups, with the group b designated as the normalized mRNA (Fig. 3), which is a combination of the NM_014496.5 and NM_001330512.1 (Fig. 2A).

3.2. hRSK4 RNAs in ensembl database

Ensembl database has a table listing two mRNAs and four noncoding RNAs of the hRSK4 gene (RPS6KA6) and has an illustration depicting the exon-intron relationship of each of these RNAs and the geographic relationships among these RNAs on the Xq21.1 (Fig. 4). However, while the table lists the latest version of the two mRNAs, the illustration seems to involve some old versions of the sequences, as the sizes of the first and last exons of the two mRNAs shown in the figures do not match the latest version of the sequences. The ENST00000262752.5 mRNA in the table corresponds with the NM_014496.5. The ENST00000620340.4 is a shorter variant of the NM_001330512.1, as its first exon is the last 81 Nts of the 3rd exon of the NM_001330512.1, meaning that it lacks the first 258 Nts, and starts with the ATG start codon, of the NM_001330512.1, thus lacking a 5' untranslational region for the translation of the NM_001330512.1-encoded protein (Supplementary Document).

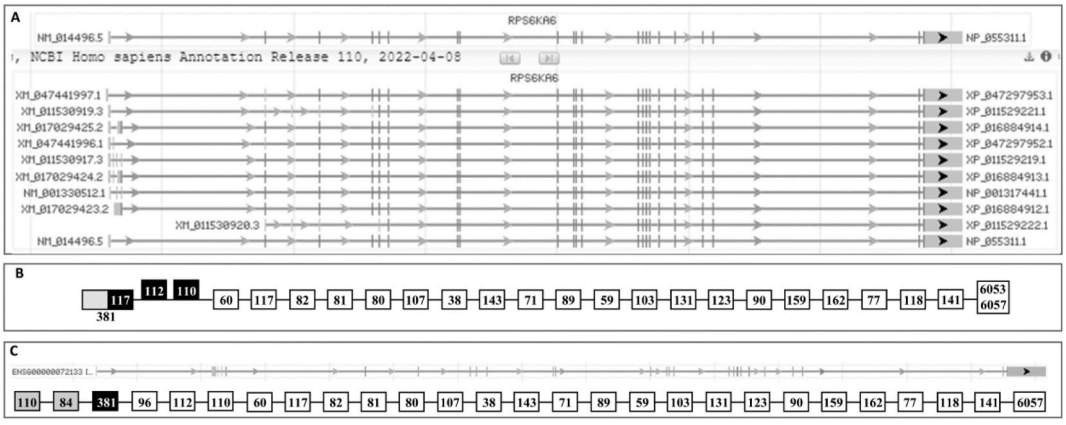


Fig. 2. The NCBI-illustrated hRSK4 mRNA variants. **A**: An image from NCBI database illustrating the exon-intron relationships of the ten mRNA variants. Each short bar or box denotes an exon while each hyphen connecting 2 bars or boxes indicates an intron, with arrows pointing to the 5′-3′ direction. Note that NCBI puts the NM_014496.5 on the top to project its Wt status and that the ten mRNAs differ mainly at the sites of transcriptional initiation and at the first exon(s). **B**: Depiction of the relationship between the NM_014496.5 and the NM_001330512.1. Boxes represent exons with the number inside or above a box indicating the number of Nts the exon has, whereas the hyphens between two boxes represent introns. The first exon (with its 5′ part grey-shaded) of the NM_014496.5 has 381 Nts while its last 117 Nts (black-shaded) constitute the first exon of the NM_001330512.1. The 2nd and 3rd exons (black-shaded) of the NM_001330512.1 are derived from the first intron of the NM_014496.5. The last exon of the NM_014496.5 and NM_001330512.1 has 6053 Nts and 6057 Nts, respectively. **C**: An image from NCBI database showing an hRSK4 mRNA that is assembled by NCBI with all 26 exons of the hRSK4 gene (ENSG00000072133) collected by Ensembl. We sketch each exon's number of Nts in this assembled mRNA. Note that its first two exons (grey-shaded) do not exist in any of the ten NCBI-listed mRNAs while its 3rd exon (black-shaded) is the first one of the NM_014496.5.

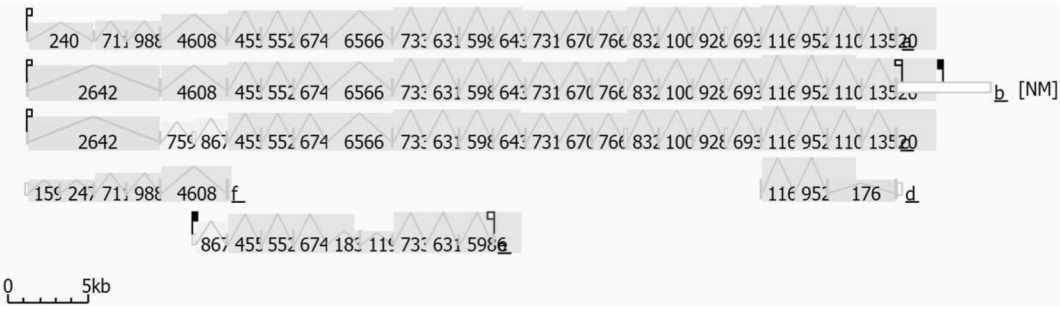


Fig. 3. An illustration from "www.aceview.org" showing the a, b, c, d, e, and f groups of hRSK4 ESTs collected by NCBI. Note that group b was then annotated as the normalized mRNA (NM), which differs from the NM_014496.5 and NM_001330512.1 shown in Fig. 2B. Mention should be made that the original image at the NCBI website already has the minor overlapping of some numbers seen in the figure.

Transcript ID	Name	bp	Protein	Biotype	CCDS	UniProt Match
ENST00000262752.5	RPS6KA6-201	8465	745aa	Protein coding	CCDS14451	Q9UK32-1
ENST00000620340.4	RPS6KA6-204	8169	745aa	Protein coding	CCDS83480	Q9UK32-2
ENST00000495332.1	RPS6KA6-203	604	No protein	Processed transcript		-
ENST00000460730.1	RPS6KA6-202	575	No protein	Processed transcript		-
ENST00000699864.1	RPS6KA6-206	170	No protein	Processed transcript		-
ENST00000699863.1	RPS6KA6-205	144	No protein	Processed transcript		-

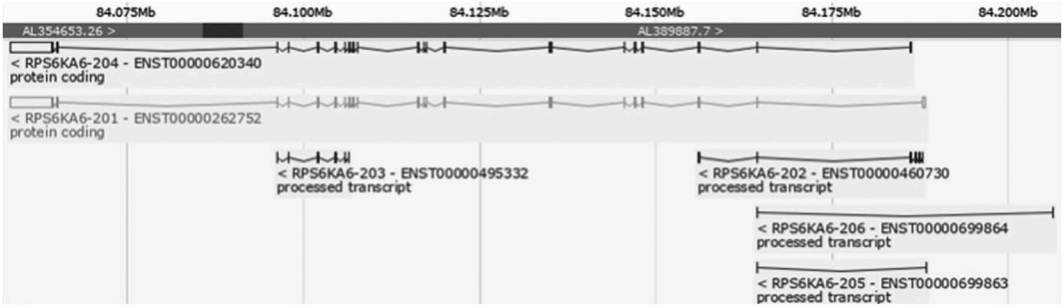


Fig. 4. The hRSK4 RNAs documented in Ensembl database. A table from Ensembl lists two mRNA variants (RPS6KA6-201 and RPS6KA6-204) and four noncoding RNAs of the hRSK4 gene (RPS6KA6). An illustration from Ensembl shows relationships among these six RNA sequences and exon-intron relationships within each RNA. However, the two mRNAs shown in the illustration seem to be an earlier version, since they differ in length from the corresponding mRNA in the table.

3.3. Unlisted hRSK4 mRNAs reported previously by us

At the time of the early 2010s when we determined hRSK4 transcripts [34], NCBI listed only one hRSK4 mRNA, i.e. the NM_014496.2 that was later updated to the NM_014496.4 that had 117 Nts in its first exon and 6057 Nts in its last exon, just like the NM_001330512.1, and had a poly A tail (Fig. 5).

Using a 5′RACE (Rapid Amplification of cDNA Ends) approach we identified an additional transcriptional initiation site that appeared in several 5′RACE clones [34]. This new site caused a 5′ extension of 222 Nts from the first Nt of the 117-Nt exon or 52 Nts from the first Nt of the 381-Nt exon in today's NM_014496.5 (Fig. 5). These 52 Nts and the first 106 Nts of the 381-Nt exon together constituted the first exon, which was followed by the first intron of 82 Nts. Its 2nd exon was as short as 24 Nts while the 2nd intron had only 12 Nts. Its 3rd exon was the last 157 Nts of the 381-exon in the NM_014496.5 (Fig. 5). We now rename this variant as "5EXT52" for its 5′ extension of 52 Nts (Fig. 5).

As depicted in Fig. 5, our previous studies identified several other mRNA variants as well, including the one that bypassed the whole 141-Nt penultimate exon (identified as DEL141) and another one that skipped only the first 39 Nts of the penultimate exon (dubbed as DEL39). In addition, we identified a variant that had a 96-Nt exon inserted between the 117-Nt first exon and the 112-Nt 2nd exon of the NM_001330512.1, which is now tagged as IN96. This 96-Nt exon has now appeared in the NCBI's XM_047441996.1, XM_011530917.3, and XM_017029424.2. Another variant we identified lacked the 2nd exon of 112 Nts in the NM_001330512.1, which is now coined as DEL112 (Fig. 5).

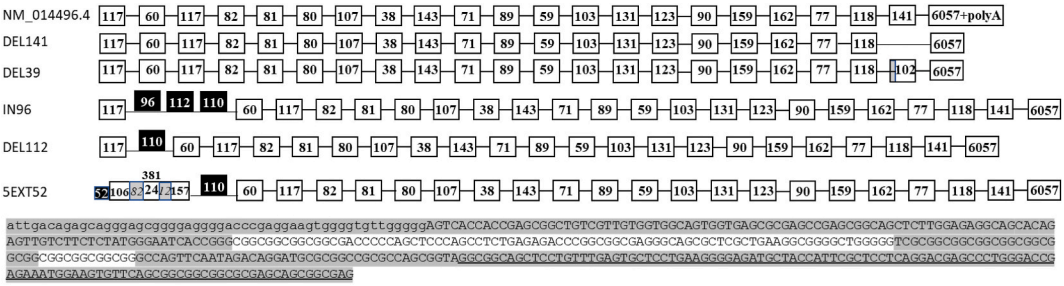


Fig. 5. The hRSK4 variants that have been reported by us previously [34] but have not yet been documented in NCBI or Ensembl. NCBI previously listed only one hRSK4 mRNA, which was updated stepwise from the NM_014496.2 until the NM_014496.4, all having 117 Nts in its first exon and 6057 Nts in its last exon and ending with a poly-A tail. We identified an mRNA variant (DEL141) that had its 141-Nt penultimate exon deleted and another variant (DEL39) that skipped only the first 39 Nts (the grey part) of the penultimate exon. A third variant we identified (IN96) had a 96-Nt exon inserted between the exons 1 and 2. A fourth variant (DEL112) we identified lacked the 112-Nt exon from the now NM_001330512.1. The 96-Nt, 112-Nt, and 110-Nt exons (black-shaded) are all derived from the intron 1 of the NM_014496.4. In addition, we identified a transcriptional initiation site that had a 5′ extension of 222 Nts from the first Nt of the 117-Nt exon 1 (underlined sequence) of the NM_014496.4 (exon 1 of the now NM_001330512.1). We rename this variant as 5EXT52 for its 5′ extension of 52 Nts from the first Nt of the 381-Nt exon 1 of the now NM_014496.5. The exons 1, 2, and 3 (shaded sequences) of this variant have 158 Nts, 24 Nts, and 157 Nts, respectively, while the introns 1 and 2 have 82 Nts and 12 Nts, respectively.

Fig. 6. The 5′ sequence of the NM_014496.5 (Wt-1) and NM_001330512.1 (Wt-2) as well as the upstream ORF for a short hRSK4 isoform in the mRNAs listed in NCBI or identified by us. **A**: The last 117 Nts (underlined) of exon 1 of Wt-1 constitute the 1st exon of Wt-2, while the last 81 (italicized) of these 117 Nts encode the first 27 AAs (with the ATG start codon shaded and boldfaced) of the Wt-1 protein (NP_055311.1). Translation of the Wt-2 mRNA incepting from this ATG stops at a TAG stop codon in exon 2, yielding a short upstream ORF (shaded sequence) encoding a 43-AA hRSK4. Translation of the NCBI-annotated hRSK4 protein (NP_00131744.1) encoded by the Wt-2 mRNA actually starts from an ATG (shaded, boldfaced and italicized) at exon 3, with the 27 AAs encoded by this exon (italic sequence) differing from the first 27 AAs of the Wt-1 protein. **B**: Six NCBI-listed variants as well as several mRNA variants we identified encompass one or two short upstream ORFs (sequences shown) encoding hRSK4 peptides (sequence shown), most of which (shaded) is identical to the N-terminus of the Wt-1 hRSK4.

3.4. ORFs encoded by NCBI- and ensembl-listed hRSK4 RNAs

We used a DNAstar software to identify all ORFs that encoded 25 or more codons in NCBI- and Ensembl-listed hRSK4 RNAs. Although the Openprot team (http://openprot.org) used a 30-codon criterion [52], we preferred the 25-codon cutoff, not only because it is automatically set by the DNAstar but also because short peptides [53–55], as short as 11 AAs [56–58], may have important functions [52,59,60].

The NM_014496.5 possesses only one ORF for hRSK4, which is a protein (NP_055311.1) of 745 amino acids (AAs) with its first 27 AAs encoded by the last 81 Nts of the first exon (Fig. 6A). The NM_001330512.1 also contains the same ATG start codon that initiates the translation of the NP_055311.1 protein, but the translation stops at the 2nd exon, engendering a 43-AA hRSK4 isoform that is partly different from the N-terminus of the NP_055311.1 (Fig. 6A and B). Translation of the NCBI-annotated hRSK4 protein encoded by the NM_001330512.1, i.e. the NP_00131744.1, actually incepts from an ATG in the 3rd exon. Since this exon 3 is unique to the NM_001330512.1 and also encodes 27 AAs, the first 27 AAs in the NP_00131744.1 and the NP_055311.1 are different (Fig. 7). As both NM_014496.5 and NN_01330512.1 are normalized mRNAs, with the former regarded by the NCBI as the principal one, we herein designate the NM_014496.5 and the NP_055311.1 as the Wt-1 mRNA and protein, respectively, and the NM_001330512.1 and the NP_00131744.1 as the Wt-2 mRNA and protein, respectively, so that they can be distinguished in the future studies.

Besides the Wt-2 mRNA, there are other five NCBI-listed mRNAs being bicistronic for hRSK4, encoding not only a long hRSK4 protein but also a short hRSK4 isoform (Fig. 6B and Table 2). Since two or three variants can produce the same short or long isoform (Figs.. 6B and 7), the ten NCBI-listed mRNAs can produce a total of nine hRSK4 protein isoforms, with the six long ones having 624–762 AAs and the three short ones having 43, 44, and 52 AAs, respectively (Table 2 and Fig. 6B). The long isoforms differ from one another mainly at the N-terminal region (Fig. 7). Moreover, all ten mRNA variants also possess additional 25–28 ORFs for non-RSK4 peptides of 24–75 AAs (Table 2).

The NCBI-assembled mRNA shown in Fig. 2C, should it really exist, is also bicistronic, yielding an upstream short ORF encoding the same 44-AA hRSK4 as the one possessed by the XM_047441996.1, XM_011530917.3, and XM_017029424.2, as well as a long downstream ORF encoding the Wt-2 protein. In addition, it encompasses 28 short ORFs for non-hRSK4 peptides.

The Ensembl-listed ENST00000262752.5 mRNA is NM_014496.5 as aforementioned, and thus encodes only the Wt-1 protein besides 25 non-hRSK4 peptides of 24–75 AAs (Table 2). The ENST00000620340.4 mRNA starts from the coding region of the Wt-2 without any 5' untranslational sequence. Therefore, although it can, theoretically, be translated to the Wt-2 protein, it remains possible that its translation actually starts from a downstream in-frame ATG locating at the 97th-99th Nts of the sequence (Supplementary Document), producing a hRSK4 isoform with 32 AAs deleted from the N-terminus of the Wt-2 protein, the same one encoded by the XM_047441996.1 (Table 2). The four non-coding RNA sequences of hRSK4 listed in Ensembl do not encode hRSK4 protein but

Fig. 7. Illustration of the differences among the long hRSK4 protein isoforms encoded by NCBI- and Ensembl-listed mRNAs. Only the different part is shown, located at the N-terminus. Note that the first 27 AAs of the protein encoded by the NM_01496.5 and ENST00000262752.5 differ from those of the protein encoded by the NM_001330512.1 and ENST00000620340.4. Also note that some mRNAs encode the same protein. Dashed lines denote a lack of the sequence.

Table 2
Open reading frames encoded by hRSK4 RNA variants[a].

	RNA variant	Number of RSK4	Number of non-RSK4
1	NM_014496.5	1 (745 AAs-Wt 1)	25 (24–75 AAs)
2	NM_001330512.1	2 (43 AAs; 745 AAs-Wt 2)	25 (24–75 AAs)
3	XM_047441997.1	1 (713 AAs)	25 (24–75 AAs)
4	XM_011530919.3	2 (52 AAs; 642 AAs)	25 (24–56 AAs)
5	XM_017029425.2	2 (43 AAs; 762 AAs)	26 (24–75 AAs)
6	XM_047441996.1	2 (44 AAs; 713 AAs)	25 (24–75 AAs)
7	XM_011530917.3	2 (44 AAs, 745 AAs)	25 (24–75 AAs)
8	XM_017029424.2	2 (44 AAs; 762 AAs)	26 (24–75 AAs)
9	XM_017029423.2	1 (762 AAs)	28 (24–75 AAs)
10	XM_011530920.3	1 (624 AAs)	25 (24–69 AAs)
11	ENST00000620340.4	1 (Wt 2 or 713 AAs)#	25 (24–75 AAs)
12	ENST00000262752.5	1 (745 AAs-Wt 1)	25 (24–75 AAs)
13	ENST00000495332.1	None	1 (91 AAs)
14	ENST00000460730.1	None	1 (30 AAs)
15	ENST00000699864.1	None	1 (37 AAs)
16	ENST00000699863.1	None	None
17	INTR1	1 (713 AAs)	26 (24–75 AAs)
18	DEL39WT 1	1 (732 AAs)	25 (24–75 AAs)
19	DEL82WT1	2 (102 AAs; 624 AAs)	23 (24–88 AAs)
20	DEL82WT2	3 (43 AAs; 102 AAs, 624 AAs)	25 (24–88 AAs)
21	DEL112WT2	2 (35 AAs, 745 AAs-Wt 2)	25 (24–75 AAs)
22	DEL141WT 1	1 (698 AAs)	25 (24–75 AAs)
23	DEL150WT1	1 (695 AAs)	25 (24–75 AAs)
24	DEL159-XM_011530920.3	1 (660 AAs)	26 (24–75 AAs)
25	DEL240	2 (43 AAs; 665 AAs)	24 (24–60 AAs)
26	DEL288-XM_011530920.3	1 (617 AAs)	26 (24–60 AAs)
27	DEL321-XM_011530920.3	1 (606 AAs)	25 (24–60 AAs)
28	DEL321WT 1	1 (638 AAs)	25 (24–75 AAs)
29	DEL357WT 1	1 (626 AAs)	25 (24–75 AAs)
30	DEL357WT2	2 (43 AAs; 626 AAs)	25 (24–75 AAs)
31	DEL420WT1	1 (605 AAs)	23 (24–60 AAs)
32	DEL420WT2	2 (43 AAs, 605 AAs)	25 (24–60 AAs)
33	DEL516WT1	1 (573 AAs)	25 (24–75 AAs)
34	DEL750	1 (463 AAs)	24 (24–136 AAs)
35	DEL1137-39WT1	1 (353 AAs)	26 (24–75 AAs)
36	DEL1356WT 1	1 (293 AAs)	25 (24–99 AAs)
37	DEL1462WT 1	2 (156 AAs; 83 AAs)	23 (24–75 AAs)
38	DEL1518WT 1	1 (239 AAs)	24 (24–73 AAs)
39	DEL1534WT1	2 (132 AAs; 83 AAs)	22 (24–75 AAs)
40	DEL1752	1 (129 AAs)	25 (24–56 AAs)
41	IN38	2 (195 AAs; 506 AAs)	25 (24–75 AAs)
42	IN96	2 (44 AAs, 745 AAs-Wt 2)	25 (24–75 AAs)
43	IN112WT2	3 (43 AAs, 55 AAs,713 AAs)	26 (24–75 AAs)
44	IN132WT1	2 (673 AAs, 83 AAs)	24 (24–75 AAs)
45	IN148WT1	2 (52 AAs, 642 AAs)	24 (24–61 AAs)
46	IN148WT2	3 (43 AAs; 52 AAs; 642 AAs)	24 (24–60 AAs)
47	IN159WT1	2 (121 AAs; 642 AAs)	25 (24–64 AAs)
48	IN159WT2	3 (43 AAs; 121 AAs; 642 AAs)	25 (24–64 AAs)
49	IN307WT 2	3 (43 AAs; 52 AAs; 642 AAs)	25 (24–60 AAs)
50	5EXT52	2 (35 AAs,745 AAs-Wt 2)	26 (24–75 AAs)

[a] Non-RSK4 peptides are those encoded by ORFs of 25 or more codons. #This Ensembl mRNA contains the whole ORF for Wt 2 protein, but it does not have any 5′ untranslational region and therefore its translation is more likely starts with a downstream in-frame ATG, yielding an RSK4 isoform lacking 32 AAs from the N-terminus of the Wt 2, the same one encoded by the XM_047441996.1.

they encode non-hRSK4 peptides (Table 2 and Supplementary Document).

3.5. Detection of novel hRSK4 variants in routinely cultured cell lines

Pairing primer F1675 with R2493 (Table 1) in RT-PCR assays could detect a 3′ region of the coding sequence shared by all ten NCBI-listed mRNAs. Pairing F35 with R830 could, theoretically, amplify a 5′-region shared by seven NCBI-listed mRNA variants, except the XM_047441997.1, XM_017029423.2, and XM_011530920.3, but the sizes of the resulting amplicons differ among the seven variants and thus can preliminarily signal which variant(s) are amplified. Although there is no way of designing a primer pair to specifically detect the Wt-1 or the Wt-2 mRNA without simultaneously amplifying some other variants, RT-PCR with the primer F156 or F198 should result in one band for the Wt-2 and the XM_011530917.3 and another band for several other variants, and will not amplify the Wt-1.

Using the F35/R830 we detected the Wt-1 mRNA, as confirmed by sequencing the RT-PCR amplicon, in the 22RV1 prostate cancer

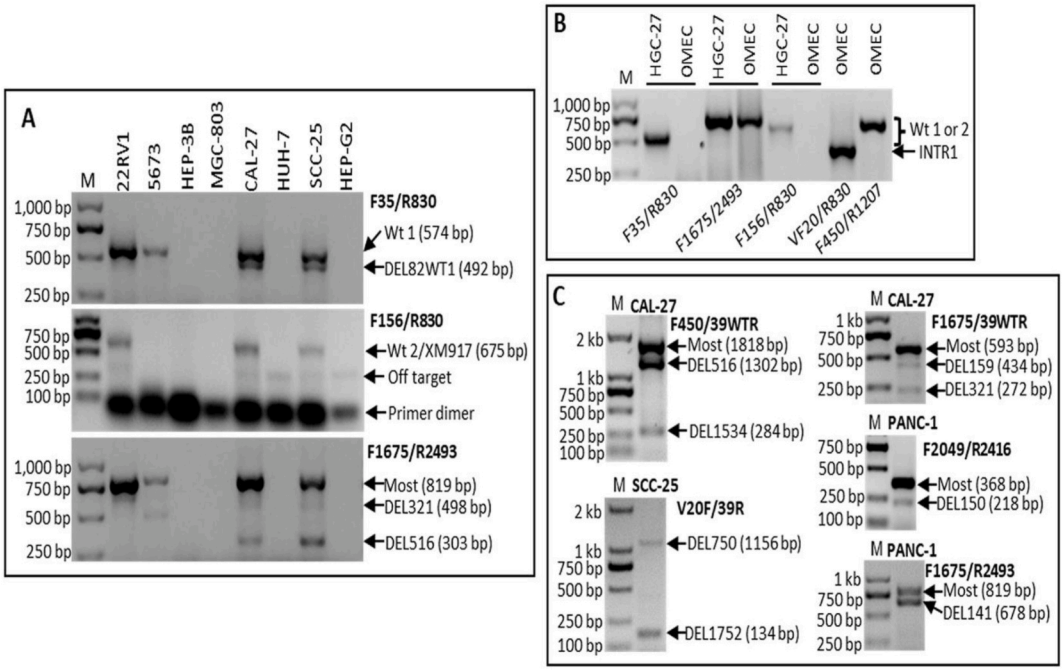


Fig. 8. RT-PCR detection of hRSK4 mRNAs in a panel of human cell lines with primer pairs covering different regions of the coding region. **A**: RT-PCR assay using the primer pair F35/R830 detects the Wt-1 and DEL82WT1 in some of the cell lines, while the primer pair F156/R830 detects the Wt-2 and/or the XM_011530917.3. RT-PCR using the F1675/R2493 amplifies a region shared by most ("Most") known variants and also two novel variants (DEL321 and DEL516). **B**: RT-PCR assays using either F35/R830 or F156/R830 cannot detect hRSK4 expression in OMEC cells but can detect expected amplicons in the HGC-27 cells included as a positive control. However, RT-PCR assays involving a forward primer located downstream of F156, either V20F, F450 or F1675, result in anticipated amplicons, suggesting that OMEC cells express an hRSK4 variant (INTR1) that is transcriptionally initiated from an intron (like the XM_011530920.3) and lacks a 5′ region. **C**: RT-PCR assays using different primer pairs as indicated detect several new hRSK4 RNA variants, besides the DEL141 reported by us previously. "M" is the marker of molecular weights.

cells, the 5637 bladder cancer cells, as well as the CAL-27 and SCC-25 oral squamous cell carcinoma cells, but not in the MGC803 human gastric cancer cells or the HUH-7, HEP-3B, and HEP-G2 hepatocellular carcinoma cells (Fig. 8A). Interestingly, F156/R830 detected the Wt-2 in most of the cell lines that expressed the Wt-1, suggesting that the two Wt mRNAs were often expressed concomitantly (Fig. 8A). The F35/R830 failed to detect the Wt-2 at the 796-bp position of the agarose gel as it was expected, which was consistent with our studies on other cell lines and tissues. The relatively lower abundance of the Wt-2 and the more preference of this primer pair to the Wt-1 might be a main reason.

Besides the Wt-1, F35/R830 also detected a smaller band in the agarose gel in some cell lines, which was confirmed by sequencing the amplicon as a variant lacking the 82-Nt exon from the Wt-1, thus termed as DEL82WT1 (Figs.. 8A and 9). In some cell lines the F1675/R2493 detected two unexpected bands in the agarose gel as well (Fig. 8A), which were confirmed by sequencing as novel variants with a deletion of 321 Nts and 516 Nts, respectively, thus tagged respectively as the DEL321 and DEL516 (Fig. 8A). The DEL321 had the 159-Nt exon 17 and the 162-Nt exon 18 excluded from the Wt-1, whereas the DEL516 had two more exons deleted, viz. the 77-Nt exon 19 and the 118-Nt exon 20 (Figs.. 8A and 9). An interesting finding was that the F1675/R2493 detected expression only in the cell lines expressing the two Wt mRNAs (Fig. 8A), which connotes that in this panel of cell lines the cells that did not express the two Wt mRNAs did not express other NCBI-listed RNA variants either. Moreover, the fact that expression was detected in only half of these eight arbitrarily selected cell lines suggests a great variation of the hRSK4 expression among different cell lines.

Neither F35/R830 nor F156/R830 detected hRSK4 in the OMEC oral mucosal epithelial cell line, although the RT-PCR detected both Wt RNAs in HGC27 human gastric cancer cells included as a positive control (Fig. 8B). However, using F450/R1207 and V20F/R830 (Table 2) that amplify regions downstream of the F35/R830-amplicon in all NCBI-listed RNA variants, we could detect strong expression in the OMEC cells as confirmed by sequencing (Fig. 8B). Since V20F is located on the first exon (the 60-Nt exon) of the XM_011530920.3, which is the exon 2 of Wt-1 and the exon 4 of Wt-2, we suspect that this cell line expresses an unreported variant that shares the same transcriptional initiation site with the XM_011530920.3 but, unlike the XM_011530920.3, contains the 82-nt exon and lacks the 148-nt exon as revealed by the PCR amplicon sizes and confirmed by sequencing. This new variant is coined herein as "INTR1" for its transcriptional initiation from an intron.

RT-PCR involving other primer pairs in different cell lines detected novel mRNA variants that were visualized in agarose gels, as shown in Fig. 8C, and confirmed by direct sequencing of the PCR amplicons: F450/39WTR detected DEL1534 that skipped, from the Wt-1, the last 34 Nts of exon 5, the first 68 Nts of exon 20, and all the exons in the between. V20F/39R detected not only DEL750 that skipped the whole region from exon 3 to exon 11 from the XM_011530920.3 but also DEL1752 that lacked, also from the

XM_011530920.3, the last 11 Nts of the 60-Nt exon, the first 29 Nts of the 118-Nt exon, and all the exons in the between. F1675/39WRT detected not only DEL321 but also DEL159 that lacked the 159-Nt exon 17 from the Wt-1, whereas F2049/R2416 detected DEL150 that lacked the 141-Nt penultimate exon and the first 9 Nts of the last exon. The previously reported DEL141 was detected as well using the F1675/R2493. These variants, which are depicted in Fig. 9 with their sequences in the Supplementary Document, might appear only in certain cell lines in certain situations, although this was not determined in this study.

3.6. Detection of novel mRNA variants in oral tissues

Using F35/R830 and F156/R830 we detected both Wt mRNAs in oral squamous cell carcinoma tissues and their adjacent uninvolved (relatively normal) tissues from four patients (Fig. 10A). Although the RNA quality and/or quantity differed slightly between the cancer tissue and its normal counterpart in these cases, the levels of Wt-1 and, to a lesser extent, Wt-2 seemed to be decreased in the cancer.

F156/R830 detected two novel variants, besides the Wt-2, in some samples as confirmed by sequencing. One had a deletion of 240 Nts from the Wt-2 (thus coined as DEL240WT2), including the last 6 Nts of the 110-Nt exon 3, the whole 60-Nt exon 4 and 117-Nt exon 5, as well as the first 57 Nts of the 82-Nt exon 6 (Fig. 9). Another variant had 420 Nts deleted from the Wt-2 (thus dubbed as DEL420WT2), including exons 5, 6, 7 and 8 (Fig. 9). These results suggest that at least some of the newly identified mRNA variants are expressed not only in vitro but also in vivo. In a separate case with only a little RNA available, we detected not only the Wt-1 but also DEL82WT1 and DEL420WT1 (Figs. 9 and 10B). Interestingly, the ratio of the Wt-1 level to the DEL82WT1 or DEL420WT1 level was lower in the cancer than in the adjacent normal tissue, due to both a decrease in the Wt-1 level and concomitant increases in the DEL82WT1 and DEL420WT1 levels in the cancer (Fig. 10B).

3.7. Effects of serum starvation and a higher ambient temperature on hRSK4 expression

Three days of complete deprival from serum of HGC-27 cells cultured at 37 °C decreased the levels of Wt-1 as detected using F35/R577 and F35/R830, when compared with the cells continuously cultured with 10% serum (Fig. 11A). However, while F198/R983 and F298/R830 detected a clear decrease in the Wt-2 level, F156/R830 detected only a small amount of Wt-2 with little effect of serum depletion on RSK4 expression in this cell line (Fig. 11A). These discrepancies may be attributed to some unknown variants detected by the F198 and F298. Reducing the serum level from 10% to 5% for three days at 37 °C only had a modest effect on both Wt mRNAs.

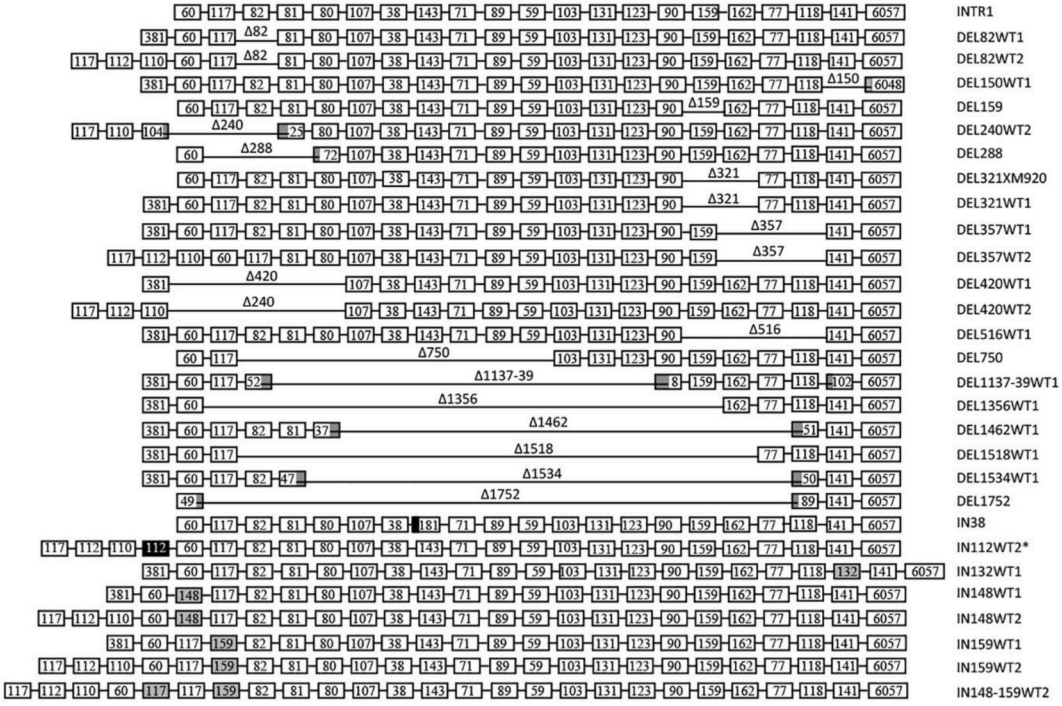


Fig. 9. Sketch of novel hRSK4 mRNA variants identified by us. Boxes represent exons while hyphens connecting boxes represent introns. The number inside a box indicates the number of Nts the exon has, whereas "Δ" indicates a sequence deletion. In the DEL150, DEL240, DEL288, DEL1137-39, DEL1462, DEL1534, and DEL1752, the grey portion of a box indicates the deleted part of the exon. In the IN38, IN132, IN148, and IN159, the shaded exon or shaded part of an exon is derived from an intron. In IN112WT2*, the inserted 112-Nt exon is derived from a 115-Nt region of the intron that, compared with the inserted 112-Nt sequence in PANC-1 cells, has not only three additional Nts (CTT or TCT) but also three mismatched nucleotides as mutations or polymorphisms (these differences are shown in the Supplementary Document).

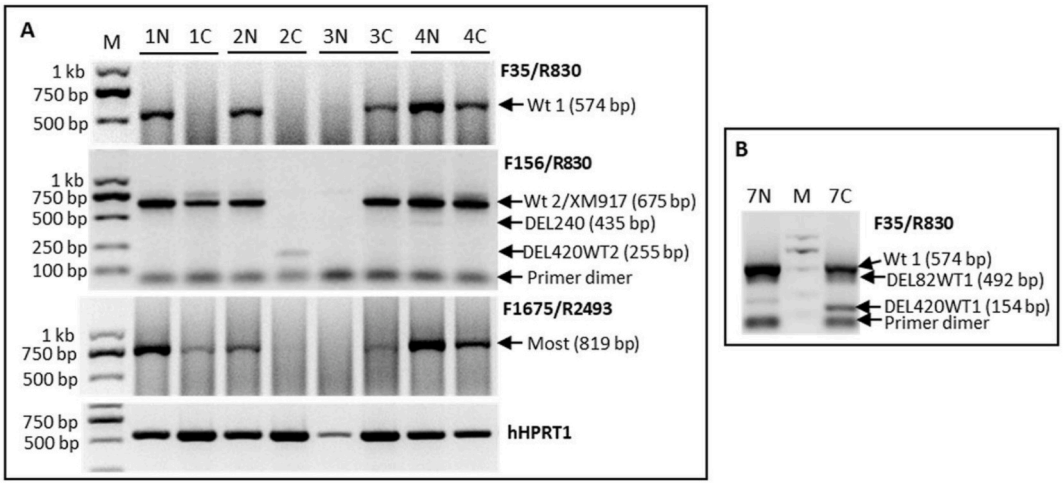


Fig. 10. Detection of hRSK4 mRNAs in oral squamous cell carcinoma tissues (C) and their relatively normal surrounding tissues (N). **A**: RT-PCR results from four paired cancer and surrounding normal tissues. cDNA of the hHPRT1 gene was used as a reference for the loading control, which shows that the RNA quality and/or quantity differs slightly between the cancer tissue and its normal counterpart in the cases 1, 2, and 4 wherein the hRSK4 level in the cancerous tissue was greatly decreased. **B**: In an additional case with only little RNA available, a novel mRNA variant, the DEL420WT1, is detected besides the Wt-1 mRNA and the DEL82WT1 variant; the ratios between different variants differ greatly in the cancerous tissue, compared with the corresponding ratios in the surrounding normal tissue. M: molecular marker.

Nevertheless, the decreases in both Wt mRNAs by serum starvation suggest that they might be required for cell proliferation that is enhanced by serum. Decreased expression could still be detected using V20F/R830 and F1675/R2638 that amplified most variants (Fig. 11A). Moreover, V20F/R830 also detected a new variant that lacked 288 Nts from the XM_011530920.3, including the 117-nt exon, the 82-Nt exon, the 81-nt exon, and the first 8 Nts of the 80-nt exon (Fig. 11A).

We have studied the effects of hyperthermia therapy on cancer and often cultured cells at 39 °C to simulate a febrile state [20,47]. We found that culture of the HGC-27 cells with 10% serum at 39 °C reduced the levels of both Wt-1 and Wt-2, as detected in most RT-PCR assays using the primers mentioned above (Fig. 11A).

In contrast to the above-described results from the HGC-27 cells, four days of serum starvation of A2780 ovarian cancer cells cultured at 37 °C increased the levels of both Wt-1 and Wt-2, as detected using F35/R830, F198/R830, and F298/R830 (Fig. 11B). Culture of this cell line either at 37 °C or at 39 °C induced the levels of Wt-1 and, more pronouncedly, Wt-2 as well, and these inductions seemed to be additive in the cells deprived of serum and cultured at 39 °C (Fig. 11B). However, RT-PCR assays using V20F/R830 that could detect most known variants did not reveal obvious changes by either serum starvation or the higher ambient temperature; this discrepancy seems to suggest that this primer pair might detect also other hRSK4 variants expression which showed different changes from what described for Wt-1 and Wt-2.

3.8. Effects of antibiotic floxacins on hRKS4 expression

A recent study of chemical screening has identified moxifloxacin and trovafloxacin, two antibiotic drugs in the floxacin family, as functional inhibitors of hRSK4 [61]. Our results of RT-PCR with F35/R830 and F156/R830 showed that moxifloxacin at a concentration range of 10–30 μM could abolish the expression of Wt-1 and Wt-2 in A2780 cells cultured at 37 °C (Fig. 11C). However, culture of this cell line at 39 °C could induce both Wt mRNAs, and this induction could counteract the inhibition of Wt-1, but not Wt-2, by the moxifloxacin (Fig. 11C), especially at a relatively lower concentration (10 μM). Interestingly, when moxifloxacin concentration was raised to 70 μM, the inhibitory effect was lost, and an even higher concentration (100 μM) started to induce both Wt-1 and Wt-2, likely because very high concentrations of moxifloxacin become stressful to the cells. Trovafloxacin did not seem to inhibit hRSK4 expression in the A2780 cells (data not shown) but, interestingly, at 5 μM it slightly inhibited both Wt mRNAs as detected using F35/R577, F35/R830, and F156/R577, and probably also some other variants as detected using F298/R830, in another human ovarian cancer cell line, SKOV-3 (Fig. 11D). Moreover, culture of SKOV-3 cells at 39 °C induced hRSK4 expression (Fig. 11D). Therefore, the inhibitory effects of these two floxacins on hRSK4 expression seem to be cell line specific and variant-specific.

3.9. Effects of clove buds on the survival and growth of pancreatic cancer cell lines

We have studied the potential therapeutic effects of clove bud, a Chinese spice and herbal medicine, on different cancer types [47,62]. By treating PANC-1 and PANC-28 cells with our clove infusion at escalating concentrations from 0.05% to 0.3% (v/v) for three days, we found that 0.2–0.3% of the clove infusion could cause a roughly 50% decrease in the live cell rate, as determined using a CCK8 assay, when the cells were cultured at 37 °C, but it needed only 0.1–0.2% of the clove to do so when the cells were cultured at 39 °C (Fig. 12A and B). Observations of the cells under the microscope reached similar estimations.

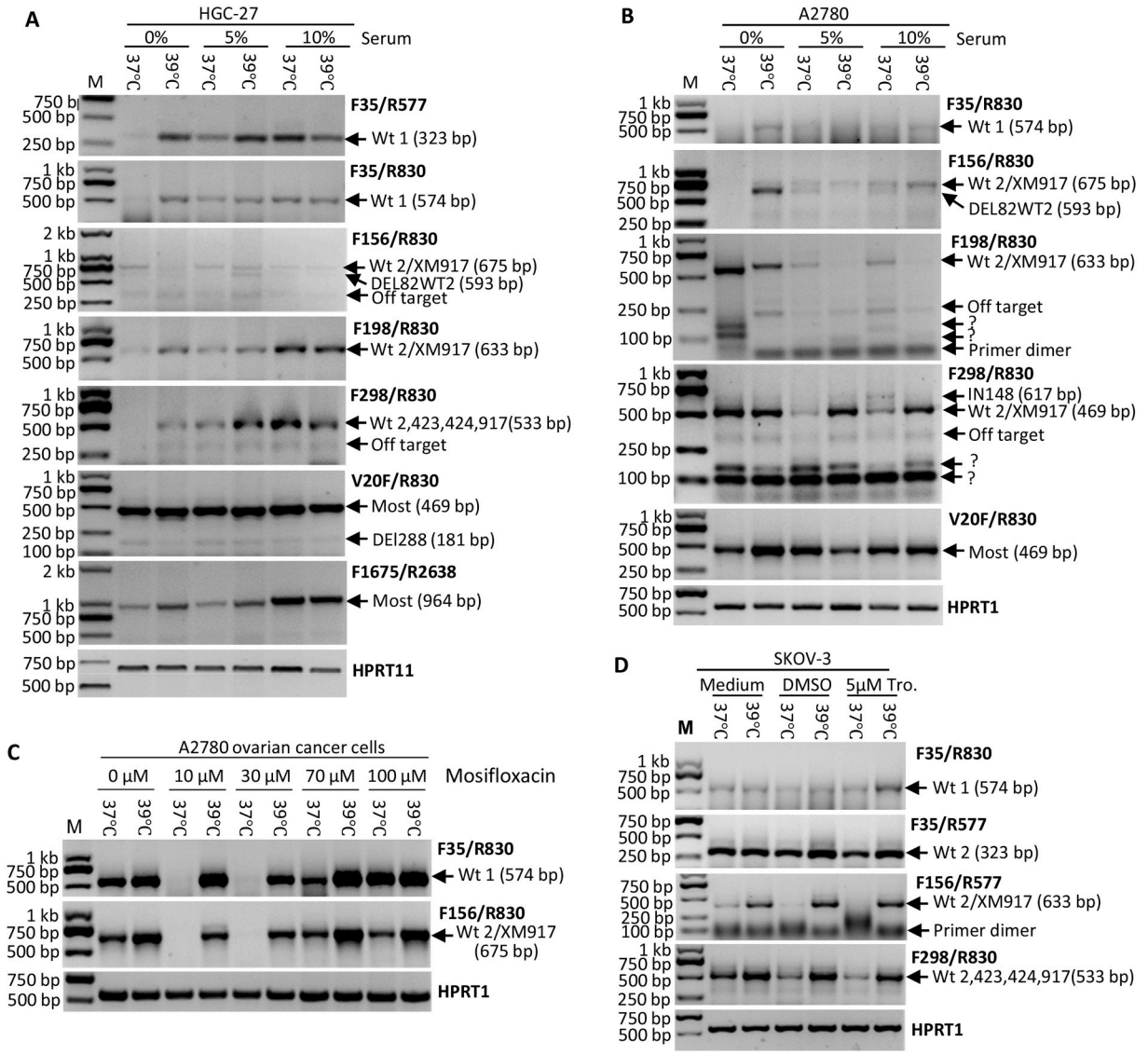


Fig. 11. Effects of serum starvation or treatment with moxifloxacin or trovafloxacin (Tro) on hRSK4 expression. **A** and **B**: RT-PCR assays of HGC-27 cells deprived of serum for three days (**A**) or A2870 cells derived of serum for four days (**B**) detect different hRSK4 variants with indicated primer pairs. Note that combining serum deprivation with a higher ambient temperature (39 °C) significantly induces the levels of both Wt-1 and Wt-2 in these two cell lines as detected by most primer pairs used, although the RT-PCR assays involving V20F or F1675 that amplifies most variants (**Most**) do not detect obvious differences among different treatments. **C**: RT-PCR assays reveal that moxifloxacin at a relatively lower concentration (10–30 μM) decreases the levels of the Wt-1 and Wt-2 in A2780 cells, but a higher ambient temperature (39 °C) can counteract this reduction. However, increase of moxifloxacin concentration to 70–100 μM starts to induce these two mRNAs, especially when the cells were cultured at 39 °C. **D**: RT-PCR assays reveal that trovafloxacin at a concentration of 5 μM can decrease the levels of the Wt-1 and Wt-2 in SKOV-3 cells. "Wt 2/XM917" means that the amplicon could be the Wt-2 and/or the XM_011530917.3. "Wt 2, 423, 424, 917" denotes that the amplicon could be any one(s) of the Wt-2, XM_017029423.2, XM_017029424.2, and XM_011530917.3. "?" denotes that the amplicon in the gels requires further determination.

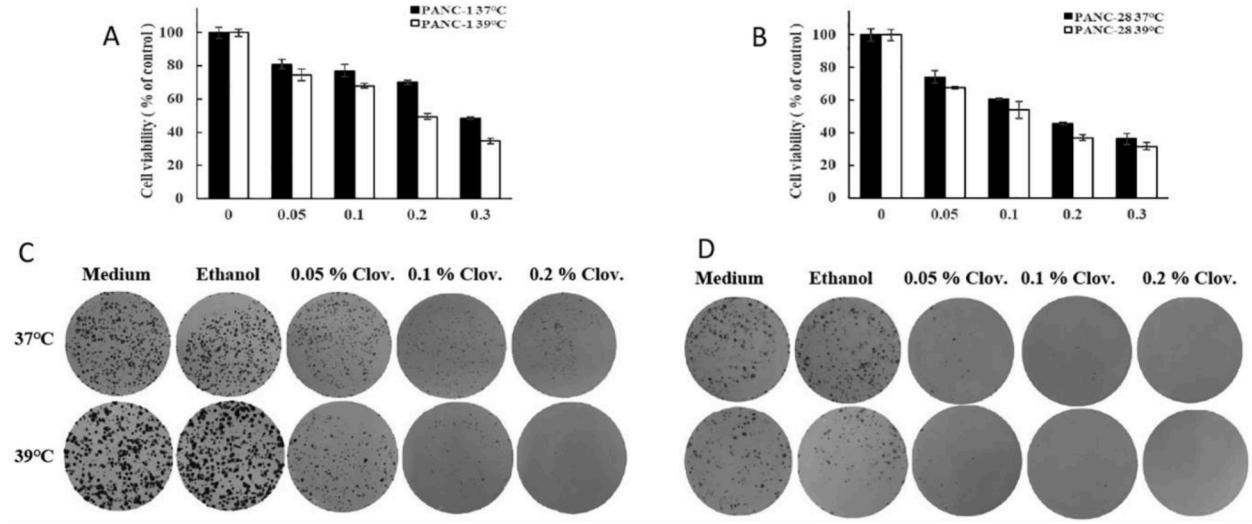


Fig. 12. Long-lasting effects of our clove infusion on the survival and growth of PANC-1 and PANC-28 cells. **A** and **B**: PANC-1 (**A**) or PANC-28 (**B**) cells cultured at 37 °C or 39 °C were treated with 0.05–0.3% of the clove and determined for the live cells using a CCK8 assay with the solvent-treated controls set as 100% survival. **C** and **D**: PANC-1 (**C**) and PANC-28 (**D**) cells seeded at 1000 cells per well in a 6-well plate and cultured at 37 °C or 39 °C were treated with the indicated concentration of clove or with DMEM (as the untreated control) or ethanol (as the solvent control). After three days of treatment the cells were cultured in a new medium without the clove or ethanol for five more days, followed by a crystal blue staining of the live cells. Note that there are notably more live PANC-1 cells at 39 °C without treatment or treated with the ethanol, whereas the PANC-28 cells showed the opposite, when compared with their counterpart at 37 °C. However, in both cell lines there are many fewer cells treated with the higher concentration (0.2%) of clove at 39 °C than at 37 °C.

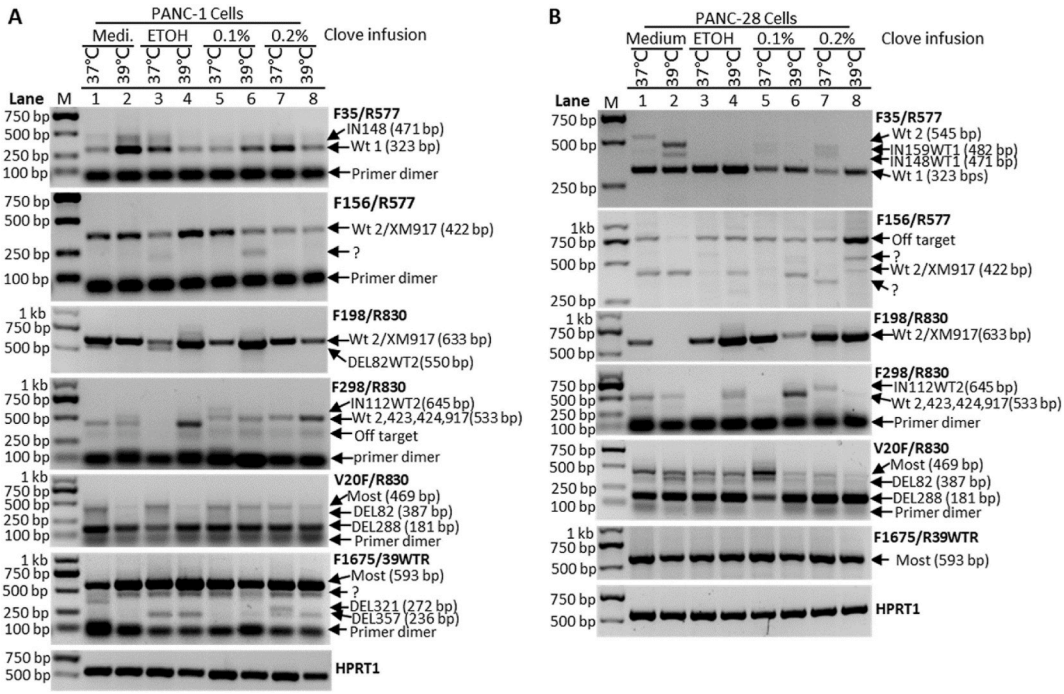


Fig. 13. Effects of our clove infusion on hRSK4 expression in PANC-1 and PANC-28 cells. PANC-1 (**A**) and PANC-28 (**B**) cells cultured at 37 °C or 39 °C were treated with 0.1% or 0.2% of the clove or with 0.2% of the solvent (95% ethanol) for three days and then evaluated for hRSK4 expression using RT-PCR assays with the indicated primer pairs. "M" denotes markers of molecular weights. 'Wt 2/XM917″ means that the amplicon can be the Wt-2 and/or the XM_011530917.3, whereas "Wt 2, 423, 424, 917" means that the amplicon can be any one(s) of the Wt-2, XM_017029423.2, XM_017029424.2, and XM_011530917.3. "?" denotes that the amplicon requires further determination.

A CCK8 assay is unsuitable in determining a growth-inhibitory or killing effect of a factor beyond several days of culture, because the untreated-control cells will grow to confluence in the 96-well plate. We therefore used a clonogenic assay to visualize the live cells treated with the clove for a longer period. When PANC-1 cells seeded at 1000 cells per well in a 6-well plate were treated, or untreated, with 0.2% ethanol (as the clove solvent) for three days at 39 °C, followed by continuous culture for five more days with a new medium without the ethanol, there were many more live cells, manifested mainly as larger colony sizes, than the cells cultured at 37 °C (Fig. 12C). This was likely because the PANC-1 cells at 39 °C grew more robustly, but not because they died less, as microscopic observation did not find obvious floating dead cells. However, when the PANC-1 cells were first treated with just a very low concentration (0.05%) of clove for three days and then cultured for additional five days without the clove, the result was reverted as there were many fewer live cells cultured at 39 °C than at 37 °C, indicating that a higher ambient temperature also facilitated the killing-effect of the clove. This phenomenon of increased killing by clove at 39 °C was also observed when the clove concentration was doubled to 0.1% and redoubled to 0.2% in the initial three-day treatment (Fig. 12C). More intriguingly, a three-day treatment with 0.05% clove only caused about 20–25% reduction in the live cell rate when determined using a CCK8 assay (Fig. 12A), in a stark contrast to the result of the "3 plus 5 days" protocol determined using a clonogenic assay shown in Fig. 12C. This discrepancy leads us to conclude that our clove infusion, even at a very low concentration, has a long-lasting cell-killing effect, lasting beyond the treatment cessation.

PANC-28 cells grew much more slowly than PANC-1 cells in routine culture, as the PANC-28 cells seeded at 1000 cells per well and cultured at 37 °C for eight days formed much fewer visualizable colonies than the PANC-1 cells (compared to the untreated cells at 37 °C in Fig. 12C with their counterparts in Fig. 12D). The untreated PANC-28 cells continuously cultured at 39 °C showed fewer live cells than at 37 °C (Fig. 12D), contrary to the PANC-1 cells (Fig. 12C). However, three-day treatment of PANC-28 cells with just 0.05% clove infusion followed by a five-day culture without the clove still resulted in much fewer colonies, similar to what was observed for the PANC-1 cells, which showed a stark contrast to the CCK8 result of only 25–30% reduction in the live cell rate at the cessation of the three-day treatment with 0.05% clove. These differences between the CCK8 and clonogenic assays at different time points suggest that the long-lasting killing effect of the clove as well as the additive effect of the higher-ambient temperature on the clove-killing similarly affect the PANC-28 cells.

3.10. Effects of a febrile temperature on hRSK4 expression in PANC-1 and PANC-28 cells

Culture of PANC-1 cells at 39 °C increased the Wt-1 expression, as detected using F35/R577, and also induced a new variant of Wt-1 that had an insertion of a 148-Nt exon between the 60-Nt exon and the 117-Nt exon, thus coined herein as IN148WT1 (Figs. 9 and 13A). In NCBI database, this 148-Nt exon appears only in the XM_011530919.3 and XM_011530920.3. Unlike Wt-1, Wt-2 detected using F156/R577 showed only a slight increase in the cells at 39 °C and did not have the insertion of the 148-Nt exon (Fig. 13A). Interestingly, in the cells cultured at 39 °C, F1675/39WTR detected a net increase in hRSK4 expression but V20F/R830 detected a significant decrease in the band of most variants and also the abundantly-expressed DEL288, while F198/R830 and F298/R830 did not detect obvious changes in hRSK4 expression (Fig. 13A). The obvious discrepancies among these RT-PCR data might partly be because each of these primer pairs had detected additional variants that had not been identified. Indeed, there were some bands shown in the agarose gels remaining to be determined (Fig. 13A). Moreover, F298/R830 detected a new mRNA variant that has a 112-Nt exon derived from intron 3 between the 110-Nt exon and the 60-Nt exon. This 112-Nt insertion actually spanned a region of 115 Nts within the third intron of the Wt-2 mRNA and, moreover, has three mismatched nucleotides compared with the genomic DNA of the PANC-1 cells (Supplementary Document). Similarly, F1675/39WTR detected a new variant that lacked a total of 357 Nts, including the 162-nt exon, the 77-nt exon, and the 118-nt exon at the 3' region.

Compared with PANC-1 cells, PANC-28 cells cultured at 39 °C showed quite different patterns of changes in hRSK4 expression. The most intriguing finding was that F35/R577 identified the Wt-2, which was a band at the 545-bp position and was confirmed by sequencing. This was the only RT-PCR result involving F35 that detected the Wt-2. Moreover, while the Wt-1 level detected with F35/R577 remained static, the IN148WT1 level was increased. A more pronounced change was the great induction of another new variant that contained an additional 159-Nt exon inserted between the 117-Nt exon and the 82-Nt exon of Wt-1; named therefore as IN159WT1 (Fig. 13B). In contrast, F198/R577 and F298/R577 detected a decrease in the Wt-2 in the cells at 39 °C, when compared with the cells at 37 °C (Fig. 13B). Moreover, V20F/R830 detected a slight decrease in the Wt form(s) but a slight increase in the DEL82 variant(s) in the PANC-28 cells at 39 °C, whereas F1675/R39WTR seemed to detect a moderate decrease in the expression in the cells at 39 °C (Fig. 13B).

3.11. Complex effects of ethanol and clove on hRSK4 expression

Treatment of PANC-1 cells at 37 °C with 0.2% ethanol included as the solvent-control for the higher concentration of our clove infusion induced the Wt-1 and IN148WT1, as detected with F35/R577, but the increases were abolished when the ethanol-treated cells were placed at 39 °C (Fig. 13A). However, treatment of the cells at 37 °C with a low concentration (0.1%) of the clove infusion, which contained small amounts of both clove extracts and ethanol, did not obviously affect the levels of the Wt-1 and IN148WT1 when compared with the untreated cells at 37 °C, but the expression was slightly increased when the cells were cultured at 39 °C (Fig. 13A). Doubling the concentration of the clove and thus also the ethanol to 0.2% raised the levels of these two mRNAs in the cells at 37 °C to the level discerned in the cells receiving ethanol alone, suggesting that the induction was attributed to the ethanol, but not the clove. Surprisingly, culture of the high-dose-treated cells at 39 °C attenuated the induction.

Ethanol alone slightly decreased the Wt-2 level in PANC-1 cells cultured at 37 °C, as detected with F156/R577, but placement of the

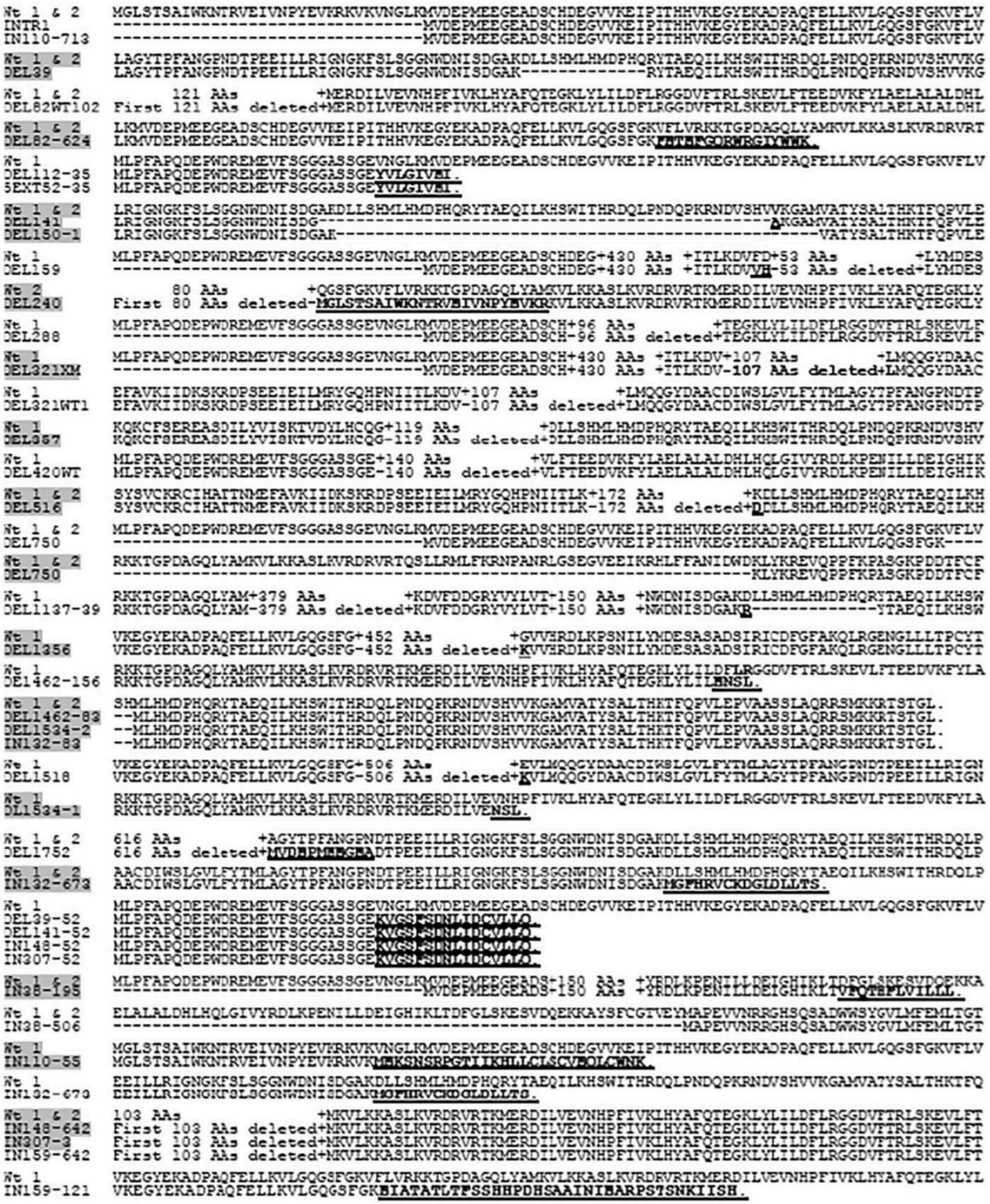


Fig. 14. Illustration of the differences between the RSK4 protein isoforms encoded by the newly-identified mRNA variants and the Wt-1 (NP_055311.1) or Wt-2 (NP_00131744.1). Only the sequence around the different region is shown. Dashed line or dashes denote deleted AAs, whereas the AAs different from those in a Wt are boldfaced and underlined. When a variant encodes two or more hRSK4 isoforms, a number is tagged after a variant's name to indicate that it has this number of AAs. Note that all short isoforms end with unmatched AAs and that some short isoforms appear in several mRNA variants.

ethanol-treated cells at 39 °C induced it (Fig. 13A). A low concentration (0.1%) of the clove did not obviously affect the Wt-2 level in the PANC-1 cells at 37 °C but decreased the level at 39 °C (Fig. 13A). Doubling the concentration of the clove and thus also the ethanol to 0.2% decreased the Wt-2 level in the cells at 37 °C or 39 °C (Fig. 13A). RT-PCR results using F198/R830, F298/R830, V20F/R830, and F1675/39WTR showed complex changes, as each of these primer pairs detected multiple variants. Collectively, this set of data suggested that in PANC-1 cells the Wt-1 might be induced by a febrile temperature and by 0.2% ethanol, whereas in most cases the Wt-2 manifested different changes to these situations of stress. Our clove infusion might not affect the Wt-1 but might decrease the Wt-2 in this cell line. Combining a higher ethanol or clove concentration with a higher ambient temperature might be too stressful to the cells, leading to complex changes in hRSK4 expression.

The PANC-28 cells treated with ethanol or clove and cultured at 37 °C or 39 °C again showed

different patterns of changes in hRSK4 expression, compared with the PANC-1 cells receiving the same treatment(s). In general, ethanol and clove treatments slightly decreased the Wt-1 but induced the Wt-2 either at 37 °C or at 39 °C (Fig. 13B). Some mRNA variants detected using F198/R830, F298/R830, V20F/R830, and F1675/39WTR also showed complex changes in the PANC-28 cells treated with ethanol or clove and cultured at 37 °C or 39 °C (Fig. 13B).

3.12. ORFs in the previously and newly identified variants

Of the five mRNA variants we identified previously (Fig. 5), the DEL141 has a deletion of 47 AAs from the C-terminal region, yielding a 698-AA hRSK4 isoform. The IN96 encodes not only the Wt-2 protein but also the same 44-AA hRSK4 peptide encoded by several XM sequences (Fig. 6). Both DEL112 and 5EXT52 variants encode not only the Wt-2 protein but also a new upstream ORF for a 35-AA hRSK4 peptide, which is the shortest hRSK4 isoform we know of (Fig. 14). All new mRNA variants identified in the present study encompass altered ORFs for novel hRSK4 protein isoforms, besides 24–26 ORFs for non-RSK4 peptides varying from 24 to 136 AAs (Table 2). The sequences of all these hRSK4 ORFs and protein isoforms are shown in the Supplementary Document, while the differences between the newly identified isoforms and the Wt-1 or Wt-2 protein are shown in Fig. 6 or Fig. 14.

The INTR1 variant skips the 148-Nt exon but retains the 82-Nt exon compared with the XM_011530920.3, which makes it encode a 713-AA hRSK4 protein that is also encoded by the XM_011530917.3 and XM_047441997.1. The DEL82 variant is bicistronic for hRSK4, having an upstream ORF for a 102-AA hRSK4 and a downstream ORF for a 624-AA hRSK4 (Table 2 and Fig. 14). The DEL112 encodes a short upstream ORF for a 35-AA hRSK4 isoform and the Wt-2 protein. The DEL141 and DEL150 have only one ORF for hRSK4, encoding a 698-AA and 695-AA hRSK4 protein, respectively. Like INTR1, DEL159 starts with the 60-Nt exon, but it skips the 159-NT exon and thus encodes only a 660-AA hRSK4. The DEL240WT2 variant retains the short upstream ORF for the 43-AA hRSK4 peptide but has a deletion of 80 AAs from the N-terminal region of the Wt-2 protein, engendering a 665-AA isoform. The DEL288 starts with the 60-Nt exon and encodes only a 617-AA hRSK4. The DEL321WT1 encodes one hRSK4 protein of 638 AAs, whereas DEL321XM920, so named because it starts with the 60-Nt exon, like the XM_011530920.3, encodes only a 606-AA hRSK4. Both DEL357WT1 and DEL357WT2 have 357 Nts deleted from the 3' region of the coding sequence and encode a 626-AA Wt-1 protein and a 626-AA Wt-2 protein, respectively, but the DEL357WT2 also encodes the 43-AA hRSK4. Both DEL420WT1 and DEL420WT2 encode an ORF that has a deletion of 140 AAs from the N-terminal region, yielding a 605-AA Wt-1 or Wt-2 protein. The DEL516WT1 variant has a deletion of 172 AAs from the C-terminal region, resulting in a 573-AA isoform of Wt-1. The DEL750 starts with the 60-Nt exon and has 750 Nts deleted from the middle region of the coding sequence, thus lacking not only the first 32 AAs from the Wt-1 protein but also 70 AAs from the middle region, yielding an hRSK4 isoform of 463 AAs. The DEL1137-39WT1 has 1137 Nts (379 AAs) deleted from the middle region and the first 39 Nts (13 AAs) deleted from the penultimate exon, yielding a 353-AA protein. The DEL1356WT1 and DEL1518WT1 also have a large region deleted from the mRNA and thus encode a 293-AA hRSK4 and a 239-AA hRSK4, respectively. The DEL1462WT1 variant lacks most of the coding region and is bicistronic for hRSK4, possessing an upstream ORF for a 156-AA hRSK4 and a downstream ORF for an 83-AA hRSK4. The DEL1534WT1 has an even larger coding sequence deleted and becomes bicistronic as well, encoding a hRSK4 of 132 AAs and another one of 83 AAs as in the DEL1462WT1. The DEL1752 lacks most of the coding region and encodes only one small hRSK4 isoform of 129 AAs.

The IN38 variant started from the 60-Nt exon; the last 38 Nts of the intron between the 38-Nt exon and the 143-Nt exon were spliced into the 143-Nt exon, making this exon larger. While the alternative initiation of transcription shortens its 5′ region and deletes the first 32 AAs from the Wt-1 or Wt-2 protein, the insertion of 38 Nts creates a stop codon in the middle of the coding region, making this IN38 variant bicistronic for an upstream hRSK4 of 195 AAs and a downstream hRSK4 of 506 AAs. The IN96 variant is bicistronic as well, possessing an upstream ORF for the same 44-AA peptide as the one seen in several XM sequences (Fig. 6), besides the ORF for the Wt-2 protein. IN112WT2 variant is a tricistronic mRNA for hRSK4, as the insertion of the 112-Nt exon into the Wt-2 mRNA creates a stop codon that breaks the long ORF into two, producing a 55-AA hRSK4 and a 713-AA hRSK4, besides the upstream 43-AA hRSK4. Similarly, in the IN148WT2 the insertion of the 148-Nt exon into the Wt 2 mRNA introduces a stop codon into its long hRSK4 ORF and breaks it into two, making the IN148WT2 tricistronic with the original upstream ORF for the 43-AA hRSK4, a new ORF for a 52-AA hRSK4 in the middle region, and another new ORF for a 642-AA hRSK4 in the downstream region. However, the IN148WT1 is only bicistronic as it lacks the 43-AA isoform. The IN307 variant has not only the 148-Nt exon but also an additional exon of 159 Nts, but it does not further change the ORFs for hRSK4. Therefore, the IN307 is also tricistronic, encoding three hRSK4 isoforms of 43 AAs, 52 AAs, and 642 AAs, respectively. In the IN132 variant, the insertion of 132 Nts at the 5' site of the penultimate exon introduces an early stop codon and makes this variant bicistronic for a long hRSK4 of 673 AAs and a downstream short hRSK4 of 83 AAs that also occurs in the DEL1462 and DEL1534 (Fig. 14).

4. Discussion

4.1. The spectrum of hRSK4 RNAs is much larger than what has been reported

The present study has identified three intron-derived novel exons, i.e. the 112-Nt, 159-Nt, and 132-Nt exons, which, together with the identification of the IN38, suggests that some introns have alternative or cryptic splice sites. In addition, quite a few variants we identified, such as the DEL150, DEL240, DEL288, DEL1137-39, etc., show splice sites within a known exon, suggesting that there are cryptic or alternative splice sites within mature mRNAs as well. Therefore, it is possible that some additional proteins detected previously on WB membranes from the cells expressing a hRSK4 cDNA construct might be derived from AS at cryptic splice sites in the construct [34]. Furthermore, it is highly possible that there are at least some unidentified RNA variants derived from different combinations of exon insertions or deletions occurring at the 5′ part with those occurring at the 3' part. The numerous RNA variants of the human titin gene produced by complex combinations of its 363 exons is one example [63,64]. Actually, this inference is bolstered not only by the deletion of the 82-Nt exon in some variants other than the XM_011530920.1 but also by the insertion of the 148-Nt exon in some variants besides the XM_011530919.3 and XM_011530920.3. The DEL1137-39 variant can also be considered as an example. It should be mentioned that, although NCBI keeps updating RNA sequences in its repository, many of the previous versions of RNAs are correct and expressed by cells, as we previously reported for the NM_014496.2 version [34] and recently reported for some old versions of HSP27 mRNA [20]. Therefore, for many genes a newer RNA version may not be a surrogate or a correction of the previous one but, instead, may be an enlargement of the RNA spectrum. This is to say that the spectrum of hRSK4 RNA variants is, in our opinion, much larger than the sum of those mentioned in this study.

For two reasons we suspect that the NM_001330512.1 designated herein as the Wt-2 is a rare RNA variant. First, it should be detected in our RT-PCR assays involving the F35 primer, but it was only detected in the PANC-28 cells using the F35/R577 primer pair (Fig. 13B). On the other hand, RT-PCR results involving F198 that is located downstream of F156 on the second exon and F298 that is located on the third exon of the NM_001330512.1 both detected abundant expression of hRSK4. Second, in Ensembl database, the corresponding RNA does not have the first two exons of the NM_001330512.1. Therefore, we surmise that the Wt-2 mRNA and protein should have two sub-variants, one of which lacks a F35-containing 5′-region and is more often expressed.

4.2. The polycistronic nature for protein isoforms is an important but rarely-mentioned property

An intriguing finding of our study is that six of the ten NCBI-listed variants and most of the newly identified ones are bicistronic or even tricistronic for hRSK4. The "bicistronic" or "polycistronic" notion is canonically referred to as an RNA that encodes two or more unrelated proteins, with each ORF belonging to an independent gene and with translation of the downstream ORF(s) mediated by Internal Ribosome Entry Sites(s) [65]. We borrow this concept to describe a mRNA encoding different protein isoforms of the same gene as seen herein for many RNA variants of the hRSK4 gene, which is a rarely-mentioned phenomenon and thus deserves further exploration for other genes' mRNAs as well. Moreover, like most coding or non-coding RNAs of other genes, all hRSK4 mRNAs encode many short ORFs for different unrelated peptides, with the longest one we identified being a 136-AA protein encoded by the DEL750 (Table 2). Although it is unclear how many of these non-RSK4 ORFs may actually be translated in certain situation(s) or cell type(s), this phenomenon reminds us that the purpose for a cell to perform AS may not necessarily be to produce another RNA variant or protein isoform but, instead, may aim to produce an unrelated RNA or protein, thus rendering a pre-RNA transcript more useful.

4.3. Different cell lines respond with AS to the same stress differently

Why there are so many AS products is still a question debated in molecular biology, and some RNA pundits consider most AS products noisy or precarious [9,15–18]. Indeed, in our long-term studies of AS using traditional approaches, we have experienced that some RT-PCR products shown as weak bands in agarose gels have been confirmed with sequencing as unreported RNA variants, but they were irreproducible in iterated experiments. Possible explanations include the abovementioned punditry that regards these RNA variants meaningless. However, an equally plausible explanation is that the difficulty to reproduce some AS data is because AS is used by the cells to respond to very subtle experimental nuances, too subtle for us to notice and thus to repeat. In the present study we have tested a wide range of stressed situations, including a febrile temperature (39 °C culture), serum depletion, treatment with moxifloxacin or trovafloxacin, and treatment with different concentrations of clove infusion or ethanol. Overarching findings are that there are not any two cell lines, even those of the same cancer origin, manifesting the same alterations of hRSK4 expression in response to the same stress, and that none of the hRSK4 mRNA variants we detected shows the same alteration in all cell lines we studied. For example, serum starvation, which is known to retard cell proliferation and even cause cell death, decreases the levels of both Wt forms in HGC-27 cells but increases their levels in A780 cells, suggesting that the roles of the two Wt mRNAs may be opposite in these two cell lines. However, several cell lines cultured at 39 °C show an induction of the Wt-1 without an obvious acceleration in cell growth. The mRNA variants detected by F198/R830 and F298/R830 showed opposite changes in different cell lines, suggesting that the Wt-2, and probably some other variants that can be amplified by these primer pairs, have cell-line specific functions as well. Furthermore, the Wt-1 or Wt-2 often shows a pattern of changes different from some smaller or larger variants expressed within the same cell line, significantly altering the ratios among the RNA variants. Two pancreatic cancer cell lines, PANC-1 and PANC-28, show different rates of cell viability when cultured at 39 °C or treated with our clove infusion, as determined using CCK8 and clonogenic assays. These differences in the cellular comport are associated with different expression spectra of hRSK4 mRNA variants. Currently, we are unable to delineate the function of each specific hRSK4 mRNA variant, in part due to the lack of primer pairs that can specifically detect the

Wt-1, Wt-2, and some other mRNA variants. However, it is clear now that the many mRNA variants, the polycistronic nature of many of these variants, the possible existence of unknown splice sites, and the possible unknown combinations of exon deletions or insertions, collectively render very sophisticated mechanisms upon the hRSK4 gene to regulate its functions. The complex ratios among different RNA variants within a cell constitute a higher level of regulation, making AS much more sophisticated and powerful and allowing cells to utilize AS as a very handy, sensitive, and versatile tool to quickly adapt to a drastic stress or to finetune themselves to a more comfortable state.

It has recently become popular to place huge efforts on performing spatial-temporal transcriptomics (or ribonomics) at the tissue or animal level, while the spectrum of RNA variants of each individual gene is still far from clear. Knowledge from the hRSK4 gene may lend clues to further characterize other genes' RNA spectra, which in turn helps the understanding of the spatial-temporal transcriptome at the organ or animal level.

4.4. Limitations

While the present study focuses on the AS of the hRSK4 gene's transcripts and has obtained some novel findings, it still has several major limitations. First, completion of RNA spectrum should include determination of whether the hRSK4 gene also has unknown transcriptional initiation sites, although existence of alternative transcriptional termination site is less likely. Second, we are still unable to prove that any of the predicated protein isoforms encoded by the newly-identified mRNAs is really expressed. Since most predicted isofroms are shorter than the Wt proteins, these studies require complex techniques, including sophisticated LC-MS/MS involving top-down procedures (but not the routine bottom-up procedure), as well as development of specific antibodies for ensuing WB and immunocytochemical or immunohistochemical approach.

4.5. Conclusions

We have identified 29 new hRSK4 mRNA variants in the present study, which, together with the five we reported previously, the ten listed in NCBI, and the two listed in Ensembl, make a total of 46 hRSK4 mRNA variants that produce a total of 35 hRSK4 protein isoforms varying from 35 to 762 AAs, besides four noncoding RNAs shown in Ensembl database. Many of the mRNA variants are bicistronic or tricistronic for hRSK4 protein isoforms. Different cell lines, even those of the same cancer origin, not only manifest a different spectrum of hRSK4 mRNA variants but also respond to the same stressed situation quite differently. On the other hand, the same stress may cause different alterations of hRSK4 variants in different cell lines, even those of the same cancer origin. These findings are novel and suggest that hRSK4 may have various functions in various physiological and pathological situations, which may be a reason why hRSK4 behaves like oncogenes in some situations but like tumor suppressors in some others. Moreover, we showed that hRSK4 has many unlisted RNA variants and some of them, such as the Wt1, cannot be specifically amplified using a single primer pair in RT-PCR. These results remind us that quantitative RT-PCR may result in unspecific data and, therefore, quantitative data need to be presented alongside with a gel image of the RT-PCR amplicon(s) yielded by the same primer pair to prove the amplicon specificity, although minor amplicon(s) may not be visible in the gel.

Ethics approval and consent to participate

All participant patients had been informed about the collection and usage of the tissues and about the possible publications, and had signed a written consent. The sample collection and usage as well as the written consent were approved by the Ethics Committee of the Stomatology Hospital, Guizhou Medical University (No.2021–26), and were abided by the approved protocol and in compliance with the principles of the Declaration of Helsinki. None of the patients' names or other personal information were disclosed through the entire study and in the present article.

Consent for publication

All authors agreed to this publication.

Funding

This work was supported by a grant (No. 82060489) to Dezhong Joshua Liao from National Natural Science Foundation of China.

Data availability statement

The data generated in the present study are included in the figures.

CRediT authorship contribution statement

Zhenwei Qin: Writing – original draft. **Jianglin Yang:** Writing – original draft. **Keyin Zhang:** Investigation. **Xia Gao:** Investigation. **Qianchuan Ran:** Investigation. **Yuanhong Xu:** Investigation. **Zhi Wang:** Investigation. **Didong Lou:** Validation. **Chunhua Huang:** Validation. **Lucas Zellmer:** Writing – review & editing. **Guangxue Meng:** Investigation. **Na Chen:** Investigation. **Hong Ma:** Conceptualization. **Zhe Wang:** Conceptualization. **Dezhong Joshua Liao:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Fred Bogott, M.D., Ph.D., at the Austin Medical Center, Mayo Clinic in Austin, Minnesota, USA, for his excellent English editing of this manuscript.

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