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Identification of novel splice variants and exons of human endothelial cell-specific chemotaxic regulator (ECSCR) by bioinformatics analysis

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Abstract

Recent discovery of biological function of endothelial cell-specific chemotaxic regulator (ECSCR), previously known as endothelial cell-specific molecule 2 (ECSM2), in modulating endothelial cell migration, apoptosis, and angiogenesis, has made it an attractive molecule in vascular research. Thus, identification of splice variants of ECSCR could provide new strategies for better understanding its roles in health and disease. In this study, we performed a series of blast searches on the human EST database with known ECSCR cDNA sequence (Variant 1), and identified additional three splice variants (Variants 2–4). When examining the ECSCR gene in the human genome assemblies, we found a large unknown region between Exons 9 and 11. By PCR amplification and sequencing, we partially mapped Exon 10 within this previously unknown region of the ECSCR gene. Taken together, in addition to previously reported human ECSCR, we identified three novel full-length splice variants potentially encoding different protein isoforms. We further defined a total of twelve exons and nearly all exon-intron boundaries of the gene, of which only eight are annotated in current public databases. Our work provides new information on gene structure and alternative splicing of the human ECSCR, which may imply its functional complexity. This undoubtedly opens new opportunities for future investigation of the biological and pathological significance of these ECSCR splice variants.

Keywords

ECSCR (ECSM2); alternative splicing; data mining; EST alignment; sequence homology; exon-intron boundary

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1. Introduction

Alternative splicing of pre-mRNA is a common posttranscriptional process utilized by eukaryotic organisms to generate multiple transcript variants from a single gene (Black, 2003; Maniatis and Tasic, 2002). Large-scale analyses of available expressed sequence tag (EST) and mRNA datasets have estimated that 35–90% of human genes can undergo alternative splicing (Brett, et al., 2000; Brett, et al., 2002; Johnson, et al., 2003; Kan, et al., 2001; Mironov, et al., 1999; Modrek, et al., 2001; Pan, et al., 2008; Wang, et al., 2008b). Many mRNA variants appear to be regulated in a tissue-specific manner (Wang, et al., 2008b) and protein isoforms may differ in function and/or subcellular localization (Kriventseva, et al., 2003; Resch, et al., 2004; Stamm, et al., 2005). Thus, alternative splicing is considered as a major mechanism contributing to protein diversity and proteome complexity (Graveley, 2001; Maniatis and Tasic, 2002; Resch, et al., 2004). Further, mounting evidence indicates that alternative splicing is not only important for normal development and differentiation (Grabowski, 2011; Jiang, et al., 2000; Lopez, 1998; Suzuki, et al., 2011), but also implicated in numerous diseases including cancer (Carstens, et al., 2000; Faustino and Cooper, 2003; Garcia-Blanco, et al., 2004; Hui, et al., 2004; Krawczak, et al., 1992; Nair, 2009; Prinos, et al., 2011; Saxena, et al., 2002).

Endothelial cell-specific chemotaxis receptor (ECSCR) (Verissimo, et al., 2009), previously known as endothelial cell-specific molecule 2 (ECSM2) (Armstrong, et al., 2008; Ma, et al., 2009), was initially identified a decade ago by in silico cloning of novel endothelial cell-specific genes (Huminiecki and Bicknell, 2000). However, its biological and cellular functions have only recently begun to be understood. We and others have independently shown that ECSCR is preferentially expressed in endothelial cells and vasculature, and is critically involved in cell migration, angiogenesis, and apoptosis (Armstrong, et al., 2008; Ikeda, et al., 2009; Ma, et al., 2009; Verma, et al., 2010). However, the precise roles of ECSCR in regulating endothelial cell functions such as motility (inhibitory or stimulatory) and angiogenesis (pro-angiogenic or anti-angiogenic) remain controversial (Armstrong, et al., 2008; Ikeda, et al., 2009; Ma, et al., 2009; Verissimo, et al., 2009; Verma, et al., 2010), and further detailed investigation is needed. Recently, we have uncovered that ECSCR is a novel endothelial cell junctional protein and promotes cell-cell adhesion (Shi, et al., 2011). Furthermore, we have demonstrated that ECSCR can attenuate basic fibroblast growth factor (bFGF)-driven cell migration through the FGF receptor (EGFR)-extracellular regulated kinase (ERK)-focal adhesion kinase (FAK) pathway, suggesting that ECSCR could be an important role in coordinating receptor tyrosine kinase (RTK)-, integrin-, and cell-cell junction-mediated signaling (Shi, et al., 2011).

The multifaceted roles of ECSCR suggested by these recent studies prompted us to further explore the possibility of alternative splicing. Given the wealth of information stored in EST databases, alignment of EST sequences to the genome and/or to other ESTs/cDNAs has been a popular and powerful means to predict alternative splicing of gene transcripts (Brett, et al., 2000; Clark and Thanaraj, 2002; Florea, 2006; Gupta, et al., 2004; Hui, et al., 2004; Mironov, et al., 1999; Modrek, et al., 2001; Wang, et al., 2008a). Very recently, we have reported the molecular characterization of two alternative splicing variants of the mouse ECSCR (Wu, et al., 2012). In this study, by employing EST-based data mining, bioinformatics, and molecular biology approaches, we identified three novel full-length mRNA variants of the human ECSCR that are predicted to encode different protein products. Moreover, we identified two novel exons (previously thought as introns), which are only included in some ECSCR isoforms. Finally, we defined nearly all exon-intron boundaries and corrected the sequence and structure of the ECSCR gene. Our work provides new information on gene structure and alternative splicing of the human ECSCR, which may imply its functional complexity.

2. Materials and methods

2.1. Data source

The human (Homo sapiens) EST database (dbEST) and the human genome database are from the NCBI website (www.ncbi.nlm.nih.gov). The human dbEST was released May 2011 and contains more than eight million human ESTs. The three assemblies of human chromosome 5 (AC_000137, Homo sapiens chromosome 5, alternate assembly HuRef, whole genome shotgun sequence; AC_000048, Homo sapiens chromosome 5, alternate assembly Hs_Celera, whole genome shotgun sequence; and NC_000005, Homo sapiens chromosome 5, GRCh37.p2 primary reference assembly) were used in this study.

2.2. Bioinformatics tools

Software products including Megablast (Zhang, et al., 2000), ClustalW2 (Larkin, et al., 2007) (www.ebi.ac.uk/Tools/msa/clustalw2), ExPASy (web.expasy.org), SOSUIsignal (Gomi, et al., 2004) and SOSUI engine (version 1.11) (Hirokawa, et al., 1998) (bp.nuap.nagoya-u.ac.jp/sosui) were used.

2.3. EST clones and sequencing correction

Human (Homo sapiens) EST clones BI834795 (dbEST ID: 9654133), AA187059 (dbEST ID: 827161), and BF526332 (dbEST ID: 6962415) were purchased from Open Biosystems, Inc. (Huntsville, Alabama, USA). Each of these EST clones was bi-directionally sequenced using forward and reverse sequencing primers on the respective vector (pCMV-SPORT6 or pBluescript SK−).

2.4. Genomic DNA extraction

Human kidney epithelial cells (HEK293) were from ATCC (Manassas, VA) and grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (all from Mediatech, Manassas, VA). Cells were harvested by scraping in cold PBS and collected by centrifugation for 10 min at 4C. The cell pellets were washed twice with cold PBS and resuspended in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) containing 1% SDS in a centrifuge tube, mixed well by shaking gently, and incubated at room temperature for 10 min. The genomic DNA was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1) for three times, followed by precipitation in 1/10 volume of sodium acetate (pH 5.2) and 3 volumes of isopropanol. The DNA pellets were washed with 70% ethanol, dried, and dissolved in TE buffer.

2.5. Polymerase chain reaction (PCR)

Genomic DNA from HEK293 cells was used as templates and primer pairs for PCRs are shown in Figure S4. Amplification was performed using Phusion high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA). Thermal cycling conditions comprised an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, and a final extension step at 72°C for 10 min. The PCR products were examined by agarose gel electrophoresis, purified, and bi-directionally sequenced.

3. Results

The strategies used to identify novel ECSCR splice variants and to define all exon-intron boundaries in this study are outlined in Figure 1.

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3.1. Initial identification of potential novel human ECSCR splice variants

To identify potential splice variants of human ECSCR, we first used the 1027bp of human ECSCR mRNA/cDNA (GenBank accession number NM_001077693) as a query sequence to search the NCBI database of human ESTs (dbEST, released May 2011 and containing more than eight million human ESTs) with Megablast. As shown in Figure 2, we obtained more than 100 hits, of which 18 ESTs have a unique missing DNA fragment (Figure 2, horizontal lines in grey between the two black arrows) compared to the query sequence. We also noticed that two ESTs (AA187059 and BF526332) harbor an insertion of DNA sequence at different positions (Figure 2, two short vertical lines in black, as indicated by arrows). We thus chose the three representative ESTs with GenBank accession numbers of BI834795, BF526332, and AA187059, respectively, for further analysis. Bi-directionally (forward and reverse) sequencing of the three EST clones identified many errors in their original sequences deposited in the GenBank (data not shown).

3.2. Identification of Variant 2 containing a 138bp deletion compared to ECSCR (Variant 1)

It is known that EST clones frequently contain sequencing errors. To minimize the influence of sequencing errors on the result, we sought to generate a consensus sequence. We extracted available sequences of the 17 out of 18 ESTs containing the unique missing DNA fragment from the NCBI database and then aligned them with the ClustalW2 program. The results indicated that they are most likely identical sequences (Supplemental Figure 1). The single nucleotide variations could be due to sequencing errors in the original ESTs deposited in the GenBank database or single-nucleotide polymorphism (SNP) within coding sequences of human ECSCR gene. Alignment of the consensus sequence (529bp, generated from Supplemental Figure 1), corrected BI834795 sequence (871bp), and the human ECSCR cDNA sequence (1027bp), revealed that the missing DNA fragment contains 138 nucleotides (Figure 3). This 138bp sequence corresponds to the unique missing DNA fragment present in the 18 ESTs shown in Figure 2. Thus, this relatively common, novel splice variant was named Variant 2 in distinction from the known human ECSCR (Variant 1). To map this 138bp deletion on the human ECSCR gene, we performed a blast search with this sequence against the three human genome assemblies (AC_000048, AC_000137, and NC_00005) focusing on the region of ECSCR. We found that the 138bp deletion is actually located within the first half of the last exon of the human ECSCR gene. Such a deletion can lead to a slight difference in the C-terminus of deduced amino acid sequences between Variants 1 and 2 (The stop codon TAA is highlighted in Figure 3).

3.3. Identification of Variants 3 containing a 108bp insertion and a 138bp deletion compared to ECSCR (Variant 1)

We next sought to obtain more information on the insertion present in the EST clone BF526332 (identified in Figure 2). By employing a similar strategy, we performed a blast search on the dbETS using BF526332 as a query sequence. The results confirmed that BF526332 indeed contains an insertion compared to ECSCR (Variant 1) (Figure 4). Notably, the other three EST clones (BP397865, AA304184, and BE177183) contain partial sequences of this insertion (Figure 4, fragments indicated by blue arrows). Multiple sequence alignment of these three ESTs, corrected BF526332, the ECSCR (Variant 1) cDNA, and the ECSCR gene (GenomicEx8) indicated that the exact length of this insertion is 108bp. Further mapping this 108bp insertion on the human ECSCR gene revealed that it is located within a region that was previously considered as an intron (Figure 4, lower panel). The extra single nucleotide present in the genomic sequence only (Figure 4, red arrow) could be a sequencing error. This novel splice variant, which is represented by BF526332 and at least other three ESTs, was designated by the name of Variant 3.

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3.4. Identification of Variants 4 containing an 119bp insertion compared to ECSCR (Variant 1)

Similarly, we performed a blast search on the dbEST using AA187059 (identified in Figure 2) as a query sequence. Our results indicated that the EST clone AA187059 indeed harbors an insertion compared to ECSCR (Variant 1) (Supplemental Figure 2). Alignment of corrected AA187059 sequences, ECSCR mRNA/cDNA (1027bp), and the ECSCR gene revealed that the length of this insertion is 119bp. Similar to the 108bp insertion described above, this insertion is also located in a region that was previously determined as an intron at the N-terminus of the ECSCR gene (data not shown).

3.5. Determination of exon-intron boundaries of the human ECSCR gene and identification of two novel exons (Exon 2 and 8)

To define the exon-intron boundaries of the human ECSCR gene, we examined the human ECSCR gene structure on the three assemblies of human chromosome 5 (AC_000137, Homo sapiens chromosome 5, alternate assembly HuRef, whole genome shotgun sequence; AC_000048, Homo sapiens chromosome 5, alternate assembly Hs_Celera, whole genome shotgun sequence; and NC_000005, Homo sapiens chromosome 5, GRCh37.p2 primary reference assembly), which are available on the NCBI website. As shown in Figure 5, a total of eight exons are annotated on the AC_000048 chromosome. However, in this assembly, the ECSCR gene contains a large, un-sequenced “intron” between the original 6th and 7th exons (Figure 5, lower panel, indicated by an orange line). The ECSCR gene is also mapped on AC_000005 chromosome and AC_000137 chromosome, respectively. In both cases, the gene is split into two parts (location A and location B) (Figure 5, upper and middle panels), which together do not cover the entire gene. Notably, the N-terminus of the ECSCR gene on AC_000137 chromosome (location A) contains an exon (Figure 5, middle panel, indicated by a black arrow), which is not identified on the AC_000048 chromosome (Figure 5, lower panel).

As outlined above, compared to ECSCR (Variant 1), Variant 2 has a 138bp deletion whereas Variant 4 harbors a 119bp insertion. In contrast, Variant 3 contains a 108bp insertion and the same 138bp deletion as in Variant 2. Considering all known exons that have been included in AC_000048 chromosome, AC_000137 chromosome (location A and location B), and NC_000005 chromosome (location A and location B) (Figure 5), we assembled the ECSCR gene by combining the three genomic sequences and aligning the full sequences of Variants 1–4 against them. By employing this strategy, we identified a total of 12 exons, of which Exons 2 and 8 are novel (Figure 6A). In addition, we found that Exon 12 (the last exon of the ECSCR gene) is split into Exon 12A and Exon 12B due to the 138bp deletion present in Variants 2 and 3 (Figure 6A). The sequence alignment results of the transcripts (containing exons only) of Variants 1–4 are shown in Figure 6B. As expected, the two newly identified exons (Exon 2 and Exon 8) are located within the regions that were previously thought as introns of the human ECSCR gene (Supplemental Figure 3). This was further confirmed by PCRs using genomic DNA extracted from human embryonic kidney (HEK) 293 cells as templates followed by bi-directional sequencing of the PCR products. The primers used in these PCRs are displayed in Supplemental Figure 4. As expected, PCRs with primer pairs Ex2L/Ex2R and Ex8L/Ex8R resulted in specific products of ~650bp (containing Exon 2) and ~500bp (containing Exon 8), respectively (Figure 7A). In particular, the single nucleotide sequencing error in the original ECSCR gene sequence (Figure 4B, the red arrow) was confirmed by our sequencing results of the ~500bp PCR product containing Exon 8 (Figure 7B).

In an attempt to map Exon 10, we performed a blast search on all human genome assemblies. However, we failed to identify its exact locus. Since there exists a large un-

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sequenced “intron” in the original ECSCR gene on the AC_000048 chromosome (Figure 5, orange line), we reasoned whether Exon 10 could be located within this unknown region. Thus, we designed primers on Exons 9, 10 and 11 and used different combinations of primers for PCRs. We obtained two specific DNA products of ~250bp and ~240bp (Figure 7C) from PCRs using primer pairs Ex10L/Ex10R1 and Ex10L/Ex10R2 (Supplemental Figure 4), respectively. Sequencing of these two PCR fragments revealed that both contain the region spanning the junction of Exon 9-Intron 9, the entire Intron 9, and the junction of Intron 9-Exon 10. The sequences of these regions have been updated in Supplemental Figure 3. However, we were not able to obtain specific products from PCRs using the primer pairs on Exon 9/Exon 11 and Exon 10/Exon 11 (data not shown). One possibility is that this previously un-sequenced region (Figure 5, orange line) contains extremely high GC contents or other secondary structures, which resulted in the failure of PCRs. Nevertheless, our results showed that Exon 10 is indeed located after Intron 9. The sequences of Exon 9 through Exon 10 have been obtained and validated.

3.6. Predicted protein sequences of the four full-length ECSCR variants (Variants 1–4)

Sequencing results of the ESTs BI834795 (Variant 2), BF526332 (Variant 3), and AA187059 (Variant 4) and their alignment with ECSCR (Variant 1) showed that the three EST clones contain full-length cDNAs (Figure 6B). We thus performed in silico translation of these cDNAs. As shown in Figure 8, the four variants share a very high amino acid homology. The slight differences between Variants 1 and 2 are the last two residues at their C-termini (Figure 8), which are resulted from the alternative 3′ splicing event (skipping of Exon 12A in Variant 2, see Figure 6). Notably, compared to the other three variants, Variant 3 has an extra 36 amino acid segment (Figure 8) due to the inclusion of Exon 8 (Figure 6). Interestingly, this insertion occurs in the beginning of the transmembrane domain. Variant 4 is the only one of the four splice variants that contains Exon 2 (Figure 6). There are two possibile in silico translations of Variant 4 transcript. If using the open reading frame (ORF) with the original start codon of ATG in Exon 1 like the other three variants, the ORF would end with the stop codon of TGA in the middle of Exon 3 (Figure 6), resulting in a predicted protein product of a total of 67 amino acids with no apparent homology with Variants 1–3 (data not shown). Alternatively, the translation of Variant 4 can start from the first ATG in Exon 3 and ends at the first stop codon of TAA in Exon 12A (Figure 6). This leads to the absence of the signal peptide in the N-terminus of the hypothetical protein of Variant 4 (Figure 8).

4. Discussion

Recently, with experimental validation of ECSCR as an endothelial cell-specific, cell-cell junctional protein and discovery of its important roles in cell-cell adhesion, motility, angiogenesis, and apoptosis (Armstrong, et al., 2008; Ikeda, et al., 2009; Ma, et al., 2009; Shi, et al., 2011; Verma, et al., 2010), ECSCR has been an interesting molecule in vascular biology research. Complete information regarding its transcriptional and posttranscriptional regulation is urgently needed to further understand the precise function of ECSCR. Alternative splicing contributes significantly to the complexity of the human transcriptome and proteome, and is a common mechanism of posttranscriptional regulation, which occurs in averagely one-half of the human genes (Brett, et al., 2002; Johnson, et al., 2003; Modrek, et al., 2001). Very recently, we have identified and characterized two alternative splicing variants of the mouse ECSCR (Wu, et al., 2012). However, the alternative splicing events of the human ECSCR gene are yet to be investigated. Complete sequencing of the human genome and public availability of EST databases have made this possible. In this study, we combine computational and experimental methods to identify splice variants of ECSCR, define nearly all exon-intron boundaries, and update the information on the human ECSCR gene.

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It is known that a gene in higher eukaryotes consists of exons and introns. The removal of introns and the joining of exons by RNA splicing is an essential step in generation of mature, functional RNA product of a gene. Alternative splicing events can assemble different combinations of exons to produce mRNA variants with distinct protein coding potentials. From a gene structure standpoint, alternative splicing events can be classified into five common types, which include exon skipping/inclusion, alternative 3′ splice sites, alternative 5′ splice sites, mutually exclusive exons, and intron retention (Cartegni, et al., 2002). As for the human ECSCR gene, we found that Exon 12A (the first half of the last exon) is spliced out in Variants 2 and 3 whereas the intact Exon 12 (including 12A and 12B) are retained in Variants 1 and 4. This represents the type of alternative 3′ splice sites. We also showed that Exon 2 and Exon 8 are present only in Variant 4 and Variant 3, respectively, which are good examples of exon skipping/inclusion events. Since ESTs are generally derived from mature spliced mRNA populations, our data obtained in this study through combined methods of EST-based data mining, PCR, and sequencing demonstrates the mRNA diversity from the single ECSCR gene.

Alignment of predicted amino acid sequences from the four full-length transcript variants of ECSCR indicates a high homology among them (Figure 8). For Variant 4, inclusion of 119bp Exon 2 in its transcript leads to a shift of the ORF and translation ending at the stop codon of TGA in the middle of Exon 3 if the same start codon (ATG) in Exon 1 is used as in Variants 1–3 (Figure 6B). Alternatively, utilization of the second ATG in Exon 3 results in a putative protein short of the signal peptide consisting of first 27 amino acids at the N-terminus. In this case, Variant 4 is identical to the mature ECSCR (Variant 1). Due to the inclusion of 108bp Exon 8, Variant 3 shows the least similarity to others. It is noted that the only difference between Variant 1 and Variant 2 occurs within the last two amino acid residues that are resulted from the alternative 3′ splicing event (intact Exon 12 versus Exon 12B only). This implies that the function of ECSCR Variants 1 and 2 could be very similar. This is somewhat surprising. Of all the blast hits biased towards the 3′ ends of ECSCR transcripts, approximately two-thirds of the ESTs resemble Variant 1 (ECSCR) containing the intact Exon 12 and the remaining one-third are similar to Variant 2 in which the Exon 12A is skipped (Figure 2). Because ESTs generally originate from random sequencing efforts, the number of ESTs corresponding to a specific variant in the dbEST is likely representative of its expression level. Thus, it is reasonable to conclude that Variants 1 and 2 are the two common isoforms of ECSCR. If this is true, one may ask why the two variants share such a high homology (nearly 98% of amino acid identity) and what the biological and functional significance is. There are several explanations. Firstly, the coding sequences for the last two residues in Variant 1 and Variant 2 correspond to the first 6 nucleotides in Exon 12A and the first 3 nucleotides in Exon 12B (Figure 6B). Thus, the major difference between the two transcript variants is located within alternative 3′ untranslated regions (UTRs). In fact, in addition to the above-mentioned five common types of alternative splicing events, alternative 5′ and 3′ UTRs are often encountered (Florea, 2006). However, they are generally harder to identify and characterize because of the intrinsic difficulty in determining the gene boundaries (Florea, 2006). The alternative 5′ and 3′ UTRs could play a role in translational control and development (Kuersten and Goodwin, 2003; Wilkie, et al., 2003). Secondly, it has been long appreciated that the alternative transcripts containing premature termination codons are targets of nonsense-mediated mRNA decay (NMD), a surveillance mechanism that selectively degrades nonsense mRNAs (Buchwald, et al., 2010; Ishigaki, et al., 2001; Kim, et al., 2001; Lewis, et al., 2003; Lykke-Andersen, 2001; Lykke-Andersen, et al., 2001; Nagy and Maquat, 1998). As already noted, splicing out Exon 12A results in the removal of the first stop codon, which makes the second stop codon in the same ORF in Exon 12B effective. Thus, it will be interesting to speculate the possibility of NMD for ECSCR, which deserves further investigation in the future. Thirdly, although the predicted protein sequences of Variants 1 and 2 are highly similar, their transcripts may

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represent different tissue origins, cell types, subcellular localizations, developmental stages, and disease specificity. Indeed, previous studies have demonstrated the spatial and temporal expression patterns of ECSCR (Armstrong, et al., 2008; Ikeda, et al., 2009; Ma, et al., 2009; Shi, et al., 2011; Verma, et al., 2010), in which the potential splice variants of ECSCR were not distinguished. Given the past research has been focused on the expression of ECSCR (Variant 1) either heterologously or endogenously, identification of the three novel full-length ECSCR isoforms (Variants 2–4) in our current study undoubtedly introduces a new dimension of studying the ECSCR gene regulation.

Although the wealth information in the dbEST and alignment of EST sequences to the human genome assemblies provide a useful, effective tool to unravel alternative transcripts, it is important to point out that there are several limitations of the EST-based methods. Firstly, lower expressed splice variant(s) may be underscored because a transcript with a higher expression level obviously has a better chance of inclusion in the dbEST. In some cases, minor splice forms can be functionally important (Geerlings, et al., 2003; Hao, et al., 1994; Wieder, et al., 1997). Secondly, alternative splicing can be highly tissue, cell type, and/or developmental stage specific and thereby detection of such splice variants really depends on the tissue type, timing and conditions when ESTs are sampled. Lower expressed variants especially have a tendency to be tissue specific (Modrek and Lee, 2003). Furthermore, many ESTs are sampled from tumor libraries. However, a predominant splice form can be expressed in normal tissue instead (Nair, 2009; Xu and Lee, 2003). Thus, the frequency of the ESTs for a specific splice variant could be biased. Thirdly, as most ESTs are generated from the 5′ or the 3′ termini of the transcript, ESTs are biased towards underrepresentation of splice forms involving exons that are in the middle of long transcripts (Johnson, et al., 2003). Lastly, the inherent problem of error-prone sequences in ESTs can lead to false positive predictions of splice variants (Modrek and Lee, 2002; Sorek and Safer, 2003). Indeed, we re-sequenced the three ESTs representing ECSCR Variants 2–4 and found many errors in their original sequences. One simple and useful method to lower the number of EST sequencing errors is to generate a consensus sequence from multiple transcripts (Gupta, et al., 2004), as used in our study (Supplemental Figure 1). Nevertheless, to overcome these potential problems, other strategies such as non-EST based methods (Hiller, et al., 2005; Sorek, et al., 2004) should be considered in the future.

In summary, in this study, using combined computational and experimental approaches we identified at least three novel splice variants (Variants 2–4) and two previously unknown exons (Exons 2 and 8) of the human ECSCR gene. We defined nearly all exon-intron boundaries and mapped a total of 12 exons on the ECSCR gene, of which only eight have been previously annotated in public databases. To our knowledge, this is the first demonstration of alternative splicing of human ECSCR gene. The existence of multiple splice variants implies that they may contribute to the multifaceted functions of ECSCR. Our study provides the molecular foundation for future exploiting the roles of ECSCR in health and disease that likely involve transcriptional, posttranscriptional, and translational regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- We identify four human ECSCR splice variants (Variants 1–4) in this study.
- We define all twelve exons and exon-intron boundaries of human ECSCR gene.
- Exons 2 and 8 are novel and have not been previously annotated in public databases.
- Our data provides new information on ECSCR gene structure and alternative splicing.

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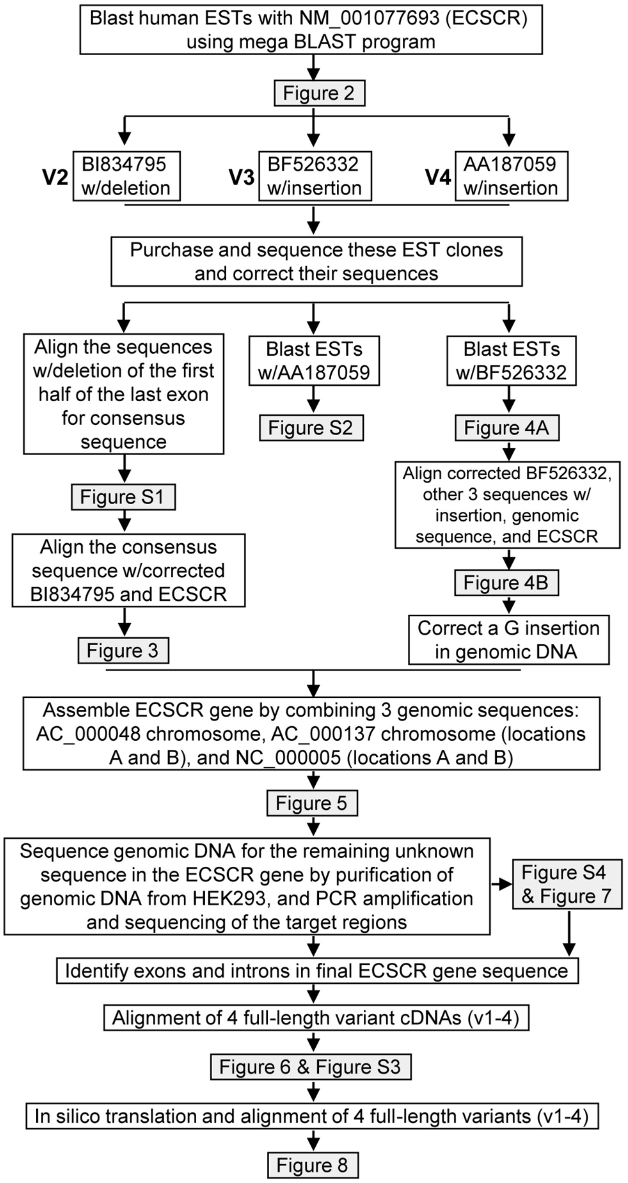
Blast human ESTs with NM_001077693 (ECSCR) using mega BLAST program
Figure 2
V2
BI834795 w/deletion
V3
BF526332 w/insertion
V4
AA187059 w/insertion
Purchase and sequence these EST clones and correct their sequences
Align the sequences w/deletion of the first half of the last exon for consensus sequence
Figure S1
Align the consensus sequence w/corrected BI834795 and ECSCR
Figure 3
Blast ESTs w/AA187059
Figure S2
Blast ESTs w/BF526332
Figure 4A
Align corrected BF526332, other 3 sequences w/ insertion, genomic sequence, and ECSCR
Figure 4B
Correct a G insertion in genomic DNA
Assemble ECSCR gene by combining 3 genomic sequences: AC_000048 chromosome, AC_000137 chromosome (locations A and B), and NC_000005 (locations A and B)
Figure 5
Sequence genomic DNA for the remaining unknown sequence in the ECSCR gene by purification of genomic DNA from HEK293, and PCR amplification and sequencing of the target regions
Figure S4 & Figure 7
Identify exons and introns in final ECSCR gene sequence
Alignment of 4 full-length variant cDNAs (v1-4)
Figure 6 & Figure S3
In silico translation and alignment of 4 full-length variants (v1-4)
Figure 8

Figure 1.

Flow chart depicting computational and experimental approaches for identification of novel human ECSCR splice variants and determination of exon-intron boundaries.

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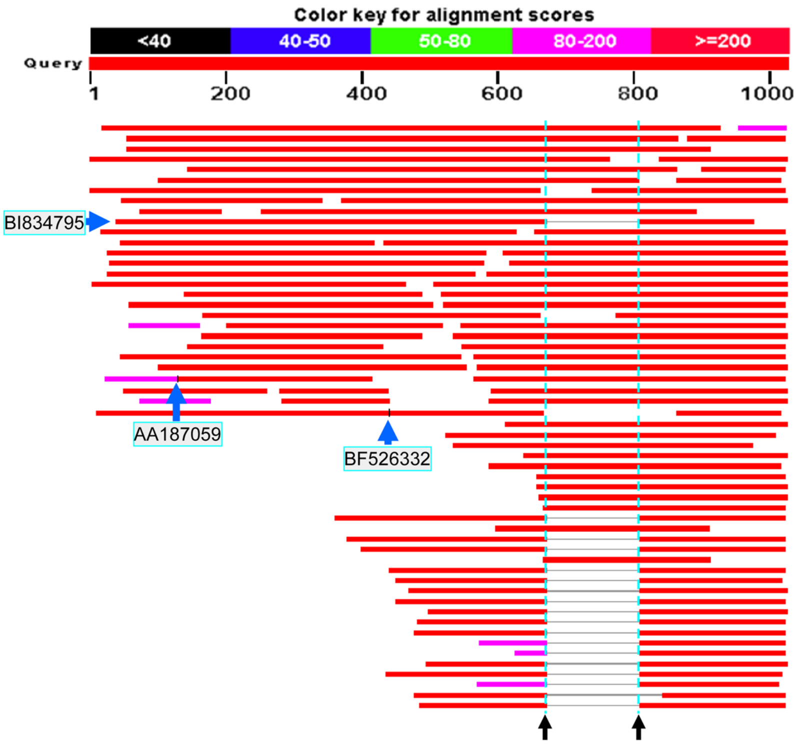

Figure 2. Blast results showing the initial discovery of novel ECSCR splice variants

A blast search on the dbEST was performed using the 1027bp human ECSCR mRNA/cDNA (GenBank accession number NM_001077693) as a query sequence. The two dotted vertical lines (in cyan) indicate the boundaries for a missing fragment (138bp) present in 18 ESTs, suggesting a relatively common splice variant. If counting the numbers of all ESTs partially or fully covering the region between the two dotted lines, this ECSCR variant form represents approximately one third of the total ESTs identified (18 out of 54). Note that the second transcript from the bottom has a longer gap. This is due to sequencing errors in the original EST, given the fact that its sequence contains multiple unknown nucleotides in this region. The short black vertical lines present in two blast hits in the middle of this map (EST clones AA187059 and BF526332) indicate that the two ESTs contain additional DNA insertions in their transcripts. They were later defined as novel Exon 2 and Exon 8 in this study.

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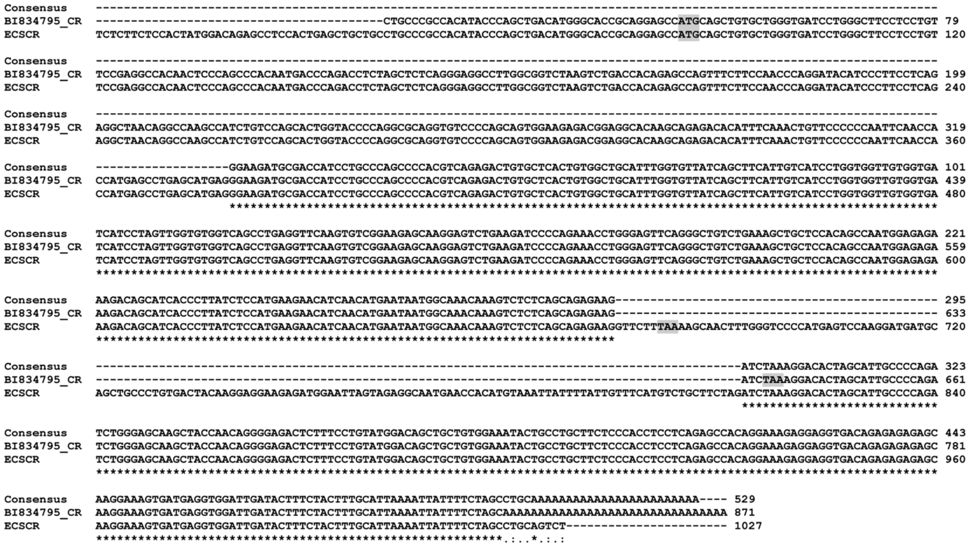

```
Consensus    ------------------------------------------------------------------------------------------------------------------------
BI834795_CR  -----------------------------------------CTGCCCGCCACATACCCAGCTGACATGGGCACCGCAGGAGCCATGCAGCTGTGCTGGGTGATCCTGGGCTTCCTCCTGT 79
ECSCR        TCTCTTCTCCACTATGGACAGAGCCTCCACTGAGCTGCTGCCTGCCCGCCACATACCCAGCTGACATGGGCACCGCAGGAGCCATGCAGCTGTGCTGGGTGATCCTGGGCTTCCTCCTGT 120

Consensus    ------------------------------------------------------------------------------------------------------------------------
BI834795_CR  TCCGAGGCCACAACTCCCAGCCCACAATGACCCAGACCTCTAGCTCTCAGGGAGGCCTTGGCGGTCTAAGTCTGACCACAGAGCCAGTTTCTTCCAACCCAGGATACATCCCTTCCTCAG 199
ECSCR        TCCGAGGCCACAACTCCCAGCCCACAATGACCCAGACCTCTAGCTCTCAGGGAGGCCTTGGCGGTCTAAGTCTGACCACAGAGCCAGTTTCTTCCAACCCAGGATACATCCCTTCCTCAG 240

Consensus    ------------------------------------------------------------------------------------------------------------------------
BI834795_CR  AGGCTAACAGGCCAAGCCATCTGTCCAGCACTGGTACCCCAGGCGCAGGTGTCCCCAGCAGTGGAAGAGACGGAGGCACAAGCAGAGACACATTTCAAACTGTTCCCCCCAATTCAACCA 319
ECSCR        AGGCTAACAGGCCAAGCCATCTGTCCAGCACTGGTACCCCAGGCGCAGGTGTCCCCAGCAGTGGAAGAGACGGAGGCACAAGCAGAGACACATTTCAAACTGTTCCCCCCAATTCAACCA 360

Consensus    -------------------GGAAGATGCGACCATCCTGCCCAGCCCCACGTCAGAGACTGTGCTCACTGTGGCTGCATTTGGTGTTATCAGCTTCATTGTCATCCTGGTGGTTGTGGTGA 101
BI834795_CR  CCATGAGCCTGAGCATGAGGGAAGATGCGACCATCCTGCCCAGCCCCACGTCAGAGACTGTGCTCACTGTGGCTGCATTTGGTGTTATCAGCTTCATTGTCATCCTGGTGGTTGTGGTGA 439
ECSCR        CCATGAGCCTGAGCATGAGGGAAGATGCGACCATCCTGCCCAGCCCCACGTCAGAGACTGTGCTCACTGTGGCTGCATTTGGTGTTATCAGCTTCATTGTCATCCTGGTGGTTGTGGTGA 480
                                *****************************************************************************************************

Consensus    TCATCCTAGTTGGTGTGGTCAGCCTGAGGTTCAAGTGTCGGAAGAGCAAGGAGTCTGAAGATCCCCAGAAACCTGGGAGTTCAGGGCTGTCTGAAAGCTGCTCCACAGCCAATGGAGAGA 221
BI834795_CR  TCATCCTAGTTGGTGTGGTCAGCCTGAGGTTCAAGTGTCGGAAGAGCAAGGAGTCTGAAGATCCCCAGAAACCTGGGAGTTCAGGGCTGTCTGAAAGCTGCTCCACAGCCAATGGAGAGA 559
ECSCR        TCATCCTAGTTGGTGTGGTCAGCCTGAGGTTCAAGTGTCGGAAGAGCAAGGAGTCTGAAGATCCCCAGAAACCTGGGAGTTCAGGGCTGTCTGAAAGCTGCTCCACAGCCAATGGAGAGA 600
             ************************************************************************************************************************

Consensus    AAGACAGCATCACCCTTATCTCCATGAAGAACATCAACATGAATAATGGCAAACAAAGTCTCTCAGCAGAGAAG---------------------------------------------- 295
BI834795_CR  AAGACAGCATCACCCTTATCTCCATGAAGAACATCAACATGAATAATGGCAAACAAAGTCTCTCAGCAGAGAAG---------------------------------------------- 633
ECSCR        AAGACAGCATCACCCTTATCTCCATGAAGAACATCAACATGAATAATGGCAAACAAAGTCTCTCAGCAGAGAAGGTTCTTTAAAAGCAACTTTGGGTCCCCATGAGTCCAAGGATGATGC 720
             **************************************************************************

Consensus    --------------------------------------------------------------------------------------------ATCTAAAGGACACTAGCATTGCCCCAGA 323
BI834795_CR  --------------------------------------------------------------------------------------------ATCTAAAGGACACTAGCATTGCCCCAGA 661
ECSCR        AGCTGCCCTGTGACTACAAGGAGGAAGAGATGGAATTAGTAGAGGCAATGAACCACATGTAAATTATTTTATTGTTTCATGTCTGCTTCTAGATCTAAAGGACACTAGCATTGCCCCAGA 840
                                                                                                         ****************************

Consensus    TCTGGGAGCAAGCTACCAACAGGGGAGACTCTTTCCTGTATGGACAGCTGCTGTGGAAATACTGCCTGCTTCTCCCACCTCCTCAGAGCCACAGGAAAGAGGAGGTGACAGAGAGAGAGC 443
BI834795_CR  TCTGGGAGCAAGCTACCAACAGGGGAGACTCTTTCCTGTATGGACAGCTGCTGTGGAAATACTGCCTGCTTCTCCCACCTCCTCAGAGCCACAGGAAAGAGGAGGTGACAGAGAGAGAGC 781
ECSCR        TCTGGGAGCAAGCTACCAACAGGGGAGACTCTTTCCTGTATGGACAGCTGCTGTGGAAATACTGCCTGCTTCTCCCACCTCCTCAGAGCCACAGGAAAGAGGAGGTGACAGAGAGAGAGC 960
             ************************************************************************************************************************

Consensus    AAGGAAAGTGATGAGGTGGATTGATACTTTCTACTTTGCATTAAAATTATTTTCTAGCCTGCAAAAAAAAAAAAAAAAAAAAAAAA---- 529
BI834795_CR  AAGGAAAGTGATGAGGTGGATTGATACTTTCTACTTTGCATTAAAATTATTTTCTAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 871
ECSCR        AAGGAAAGTGATGAGGTGGATTGATACTTTCTACTTTGCATTAAAATTATTTTCTAGCCTGCAGTCT----------------------- 1027
             **********************************************************.:..*.:.:
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Figure 3. Splicing out the 138bp DNA fragment (Exon 12A) in Variant 2

Alignment of the 529bp EST consensus sequence (generated from Figure S1), corrected sequence of EST clone BI834795 (denoted as BI834795_CR), and full-length human ECSCR mRNA/cDNA (GenBank accession number NM_001077693) reveals the actual nucleotide sequences of the unique missing DNA fragment (138bp) in the 18 ESTs (identified in Figure 2). Thus, ECSCR Variant 2 represented here by BI834795 has a skipped exon (138bp). This exon was later named Exon 12A in this study. The start codon (ATG) and stop codon (TAA) in ECSCR and BI834795 are shaded.

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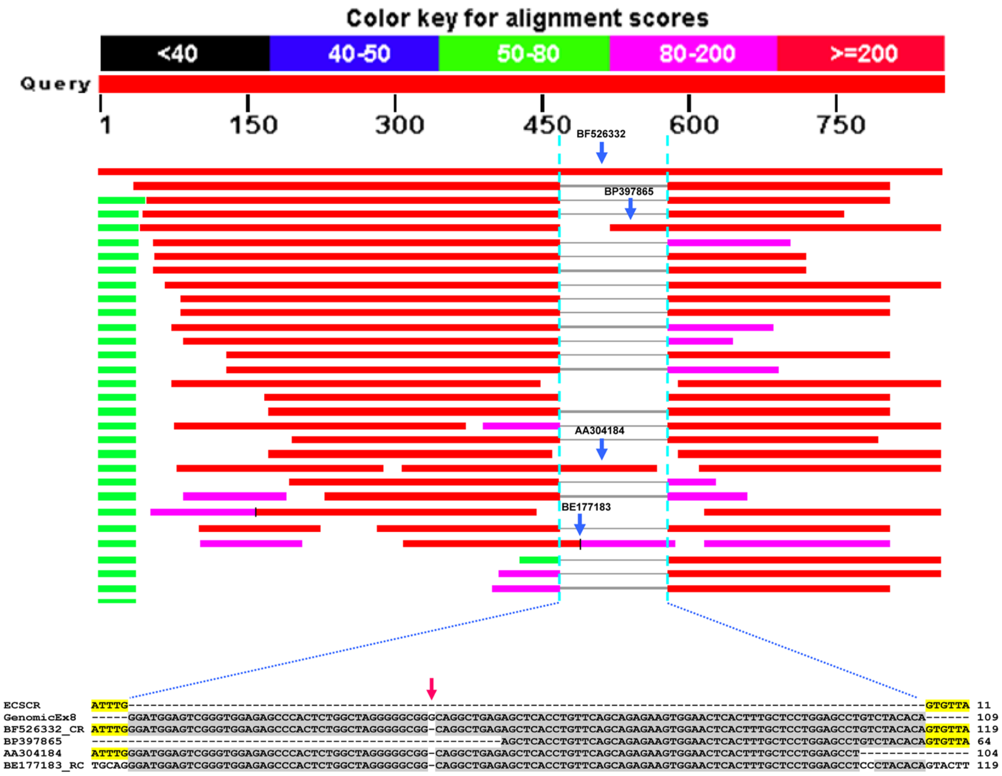

```
ECSCR        ATTTG-------------------------------------------------------------------------------------------------------------GTGTTA 11
GenomicEx8   -----GGATGGAGTCGGGTGGAGAGCCCACTCTGGCTAGGGGGCGGGCAGGCTGAGAGCTCACCTGTTCAGCAGAGAAGTGGAACTCACTTTGCTCCTGGAGCCTGTCTACACA------ 109
BF526332_CR  ATTTGGGATGGAGTCGGGTGGAGAGCCCACTCTGGCTAGGGGGCGG-CAGGCTGAGAGCTCACCTGTTCAGCAGAGAAGTGGAACTCACTTTGCTCCTGGAGCCTGTCTACACAGTGTTA 119
BP397865     --------------------------------------------------------AGCTCACCTGTTCAGCAGAGAAGTGGAACTCACTTTGCTCCTGGAGCCTGTCTACACAGTGTTA 64
AA304184     ATTTGGGATGGAGTCGGGTGGAGAGCCCACTCTGGCTAGGGGGCGG-CAGGCTGAGAGCTCACCTGTTCAGCAGAGAAGTGGAACTCACTTTGCTCCTGGAGCCT--------------- 104
BE177183_RC  TGCAGGGATGGAGTCGGGTGGAGAGCCCACTCTGGCTAGGGGGCGG-CAGGCTGAGAGCTCACCTGTTCAGCAGAGAAGTGGAACTCACTTTGCTCCTGGAGCCTCCCTACACAGTACTT 119
```

Figure 4. Blast results showing the identification of Variant 3

As noted in Figure 2, the EST clone BF526332 harbors an insertion (later named Exon 8). To confirm that this is not a random event, a blast search on the dbEST was performed using BF526332 as a query sequence. At least three additional EST clones (BP397865, AA304184, and BE177183) were identified to contain this inserted region (indicated by blue arrows), suggesting that it is a novel variant (Variant 3). Nucleotide sequence alignment of ECSCR (Variant 1), genomic region corresponding to Exon 8 (GenomicEx8), corrected BF526332 (BF526332_CR) and three ESTs (BP397865, AA304184, and BE177183) shows that Variant 3 contains an insertion of 108bp (Exon 8) highlighted in grey, which is not present in Variant 1. The red error indicates a sequencing error in the genomic DNA.

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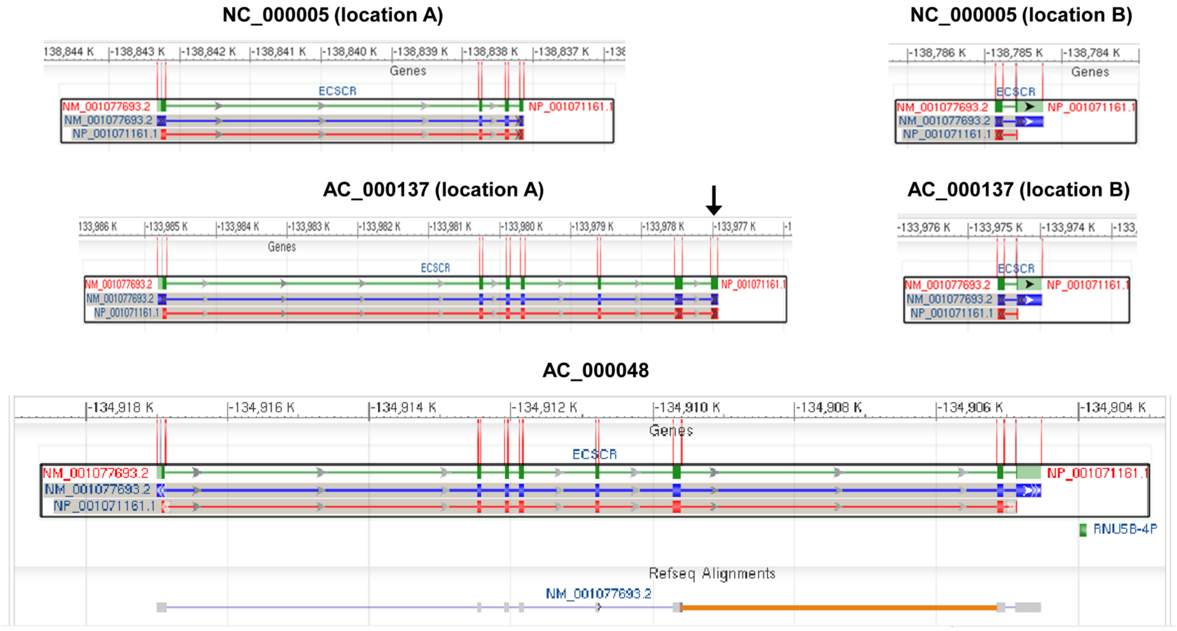

Figure 5. Original gene structure of human ECSCR

The gene structures shown here are the mapping results on the three assemblies of human chromosome 5 (AC_000137, Homo sapiens chromosome 5, alternate assembly HuRef, whole genome shotgun sequence; AC_000048, Homo sapiens chromosome 5, alternate assembly Hs_Celera, whole genome shotgun sequence; and NC_000005, Homo sapiens chromosome 5, GRCh37.p2 primary reference assembly) that are available in the NCBI databases. Green bars (the first row in each panel) represent the ECSCR gene and blue bars (the second row) show exons. The protein product is in the third row with red bars as translated gene products. Note that the last exon only encodes for two amino acid residues before the stop codon TAA (see ECSCR in Figure 3). On the AC_000048 assembly, a total of eight exons are initially defined. However, the ECSCR gene contains a large, un-sequenced region (thought as a large intron), which is indicated by the orange line. Also, note that the gene is split into two parts (location A and location B) on either AC_000137 or NC_000005 assembly, which together do not cover the entire gene. The black arrow indicates the extra exon identified only on the AC_000137 assembly (location A) but not on the AC_000048 assembly.

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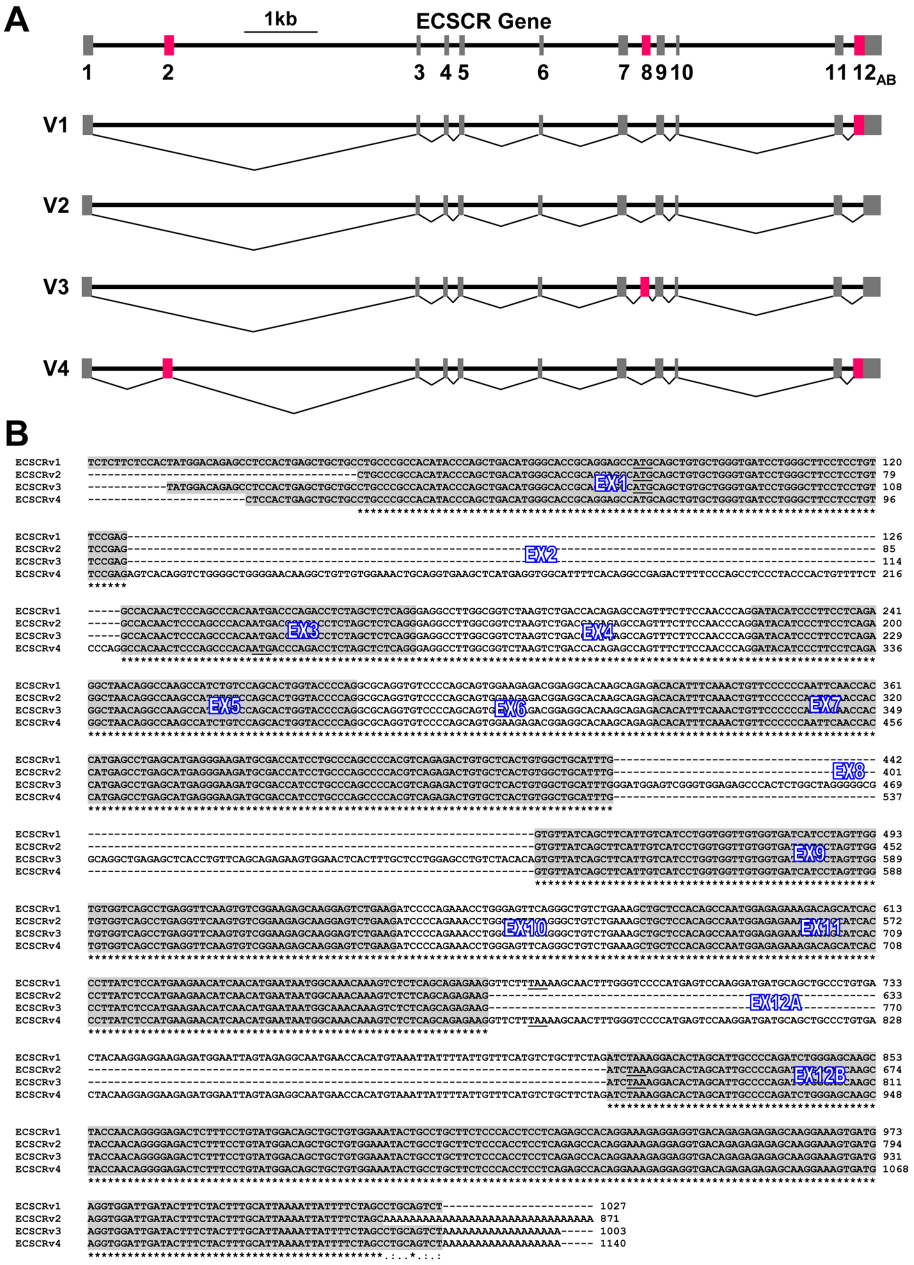

Figure 6. Reconstruction of the human ECSCR gene based on the data presented in this study

(**A**) New ECSCR gene structure and alternative splicing events. Exons are shown by closed boxes. Red boxes indicate the newly identified exons whose splicing results in four splice variants of human ECSCR. Variant 1 is the originally known ECSCR, the most common isoform. Variant 2 is a second common isoform with skipped Exon 12A compared to Variant 1. Variant 3 is an uncommon isoform including the novel Exon 8. Variant 4 is a rare isoform including the novel Exon 2. (**B**) Alignment of the complete nucleotide sequences of the four full-length ECSCR variants. A total of twelve exons (Exons 1–11, Exon 12A and Exon 12B) are marked. The start codon (ATG) and stop codon (TAA) for each splice variant are underlined. See text for details.

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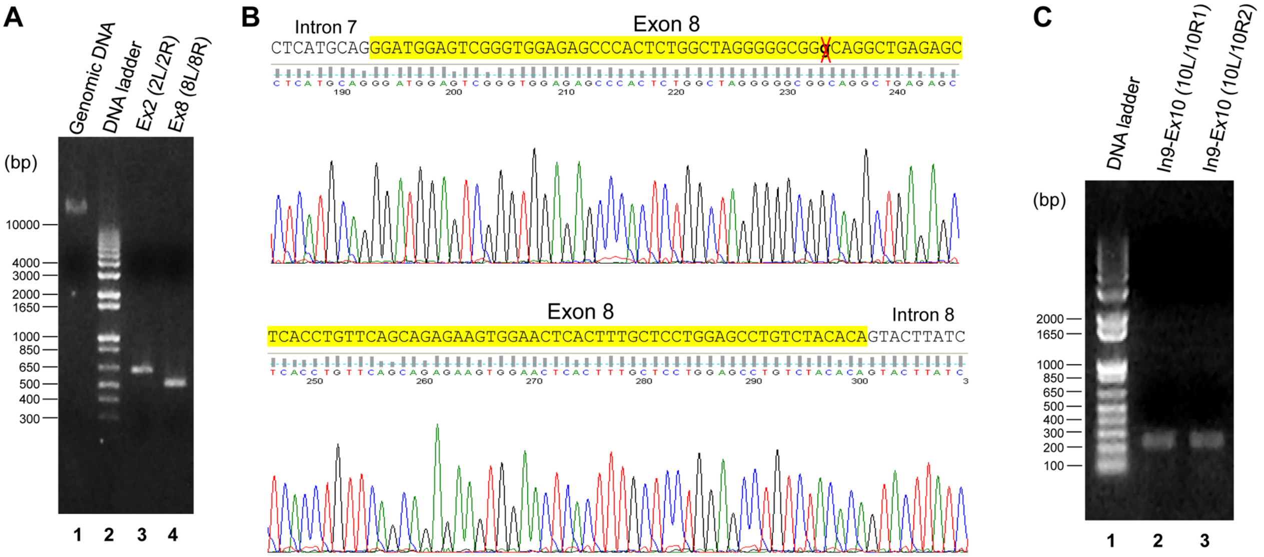
A
Genomic DNA
DNA ladder
Ex2 (2L/2R)
Ex8 (8L/8R)
(bp)
10000
4000
3000
2000
1650
1000
850
650
500
400
300
1
2
3
4
B
Intron 7
Exon 8
CTCATGCAGGGATGGAGTCGGGTGGAGAGCCCACTCTGGCTAGGGGGCGGgCAGGCTGAGAGC
C T C A T G C A G G G A T G G A G T C G G G T G G A G A G C C C A C T C T G G C T A G G G G G C G G C A G G C T G A G A G C
190
200
210
220
230
240
Exon 8
Intron 8
TCACCTGTTCAGCAGAGAAGTGGAACTCACTTTGCTCCTGGAGCCTGTCTACACAGTACTTATC
T C A C C T G T T C A G C A G A G A A G T G G A A C T C A C T T T G C T C C T G G A G C C T G T C T A C A C A G T A C T T A T C
250
260
270
280
290
300
3
C
DNA ladder
In9-Ex10 (10L/10R1)
In9-Ex10 (10L/10R2)
(bp)
2000
1650
1000
850
650
500
400
300
200
100
1
2
3

Figure 7. Experimental validation of exons 2, 8, and 10

(**A**) PCR results of the gene fragments containing Exon 2 and Exon 8. The primer pairs used in PCRs are shown in Figure S4. As revealed by agarose gel electrophoresis, the targeting sizes of specific PCR products are ~650bp (containing Exon 2, lane 3) and ~500bp (containing Exon 8, lane 4), respectively. The genomic DNA extracted from HEK293 cells (lane 1) was used as templates in the PCRs. The sizes of DNA ladder are also shown (lane 2). (**B**) Representative sequencing results of the PCR products. The PCR product containing Exon 8 (lane 4 in Panel A) was sequenced. Comparison results confirm that a single nucleotide (G) in the original ECSCR gene (marked by a red cross) is indeed a sequencing error. Also see Figure 4B. (**C**) PCR results of the gene fragments spanning the junction of Exon 9 – Intron 9, the entire Intron 9, and the junction of Intron 9 – Exon 10. The primer pairs used in PCRs are shown in Figure S4. The targeting sizes of specific PCR products using primer pairs Ex10L/Ex10R1 and Ex10L/Ex10R2 are ~250bp and ~240bp, respectively.

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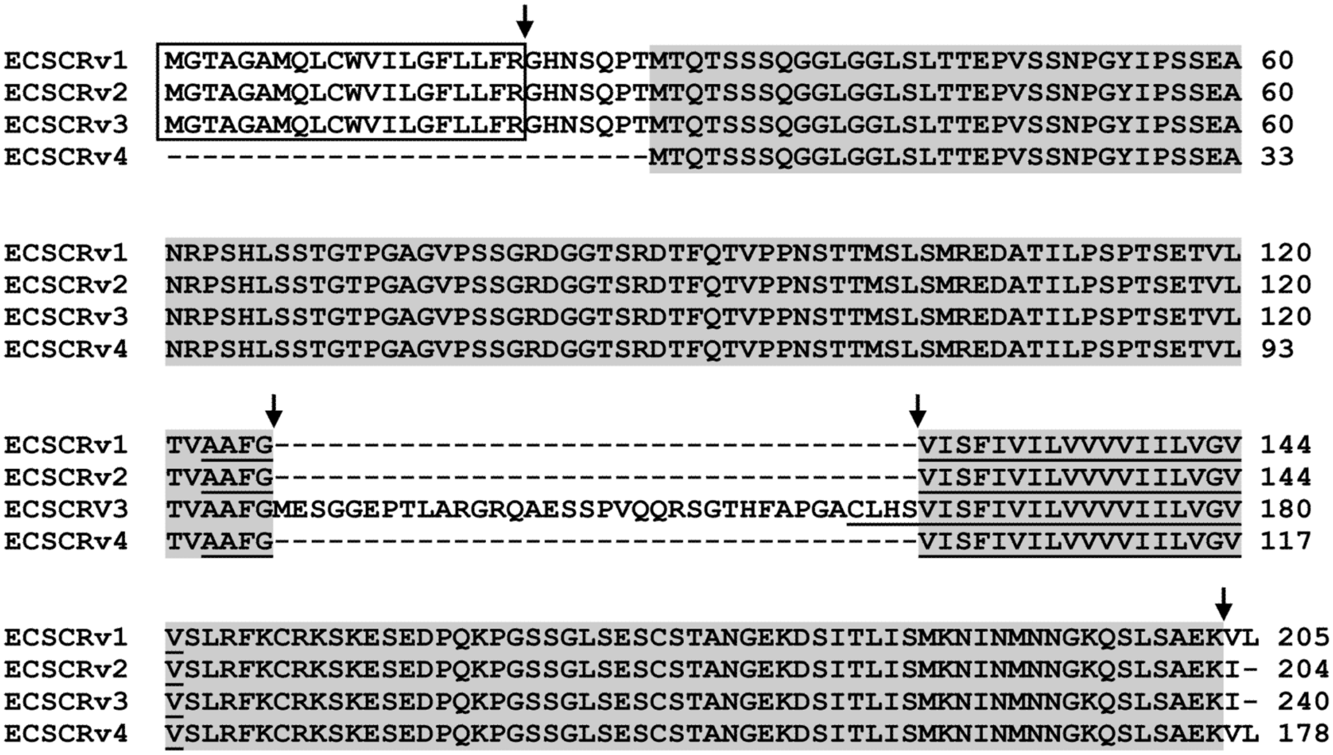
ECSCRv1 MGTAGAMQLCWVILGFLLFRGHNSQPTMTQTSSSQGGLGGLSLTTEPVSSNPGYIPSSEA 60
ECSCRv2 MGTAGAMQLCWVILGFLLFRGHNSQPTMTQTSSSQGGLGGLSLTTEPVSSNPGYIPSSEA 60
ECSCRv3 MGTAGAMQLCWVILGFLLFRGHNSQPTMTQTSSSQGGLGGLSLTTEPVSSNPGYIPSSEA 60
ECSCRv4 ---------------------------MTQTSSSQGGLGGLSLTTEPVSSNPGYIPSSEA 33
ECSCRv1 NRPSHLSSTGTPGAGVPSSGRDGGTSRDTFQTVPPNSTTMSLSMREDATILPSPTSETVL 120
ECSCRv2 NRPSHLSSTGTPGAGVPSSGRDGGTSRDTFQTVPPNSTTMSLSMREDATILPSPTSETVL 120
ECSCRv3 NRPSHLSSTGTPGAGVPSSGRDGGTSRDTFQTVPPNSTTMSLSMREDATILPSPTSETVL 120
ECSCRv4 NRPSHLSSTGTPGAGVPSSGRDGGTSRDTFQTVPPNSTTMSLSMREDATILPSPTSETVL 93
ECSCRv1 TVAAFG------------------------------------VISFIVILVVVVIILVGV 144
ECSCRv2 TVAAFG------------------------------------VISFIVILVVVVIILVGV 144
ECSCRV3 TVAAFGMESGGEPTLARGRQAESSPVQQRSGTHFAPGACLHSVISFIVILVVVVIILVGV 180
ECSCRv4 TVAAFG------------------------------------VISFIVILVVVVIILVGV 117
ECSCRv1 VSLRFKCRKSKESEDPQKPGSSGLSESCSTANGEKDSITLISMKNINMNNGKQSLSAEKVL 205
ECSCRv2 VSLRFKCRKSKESEDPQKPGSSGLSESCSTANGEKDSITLISMKNINMNNGKQSLSAEKI- 204
ECSCRv3 VSLRFKCRKSKESEDPQKPGSSGLSESCSTANGEKDSITLISMKNINMNNGKQSLSAEKI- 240
ECSCRv4 VSLRFKCRKSKESEDPQKPGSSGLSESCSTANGEKDSITLISMKNINMNNGKQSLSAEKVL 178

Figure 8. Predicted protein sequences of the four full-length ECSCR variants

The putative signal peptide is boxed. The predicted single transmembrane domain is underlined. The arrows indicate where alternative splicing occurs in the four ECSCR variants. All identical amino acids among the four variants are highlighted. See text for details.