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ROLE OF COXSACKIE AND ADENOVIRUS RECEPTOR (CAR) IN HUMAN DISEASE

Specific detection of CAR-SIV isoform using nucleic acid assays

Faculty of Medicine and Health Technology
Master's thesis (Medicine)
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ABSTRACT

Parkkila Mikael: “Role of coxsackie and adenovirus receptor (CAR) in human disease”
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Background: The Coxsackievirus and adenovirus receptor (CAR) is a protein with both soluble and transmembrane isoforms which coxsackie B viruses (CVB) and many adenoviruses have been shown to bind to. In addition with viral binding, CAR functions as a cell adhesion protein in the tight junctions of human polarized epithelial cells, where significant differences in the cellular localization of the transmembrane CAR isoforms have been presented. In non-polarized cells, such a clear difference is not present. In beta cells of the pancreas, the seven-exon isoform (CAR-SIV) localizes to insulin secretory granules. Ultimately, the functions of CAR and its different isoforms are still unclear. In recent years, a connection between enterovirus infections, especially CVB infections, and Type 1 diabetes (T1D) has been observed. The pathophysiological factors behind this are not known, but CAR, as a receptor for CVBs, presents as an important research topic.

Aims: The aims of this work are: 1) To gather an overview on CAR and its physiological functions and connections to diseases, 2) to search information about polymerase chain reaction (PCR) primers previously used in the analysis of CAR, and 3) based on these findings develop a new isoform specific set of primers for the analysis of CAR-SIV expression in cells.

Methods: A literature search was performed on PubMed, and six publications with possibly suitable PCR primers were identified. Information on the primers and probes used were collected, where available, and compared. Criteria for primer localization were the exon splice site between exons six and seven for the forward primer, and the CAR-SIV specific part of exon seven for the reverse primer. In the end, sequences for the forward primer and probe were chosen based on previous literature. Two novel reverse primers were designed for this study. RNA was extracted from Chinese hamster ovary (CHO) cells transfected with human CAR. Non-transfected CHO cells were used as negative controls. Real-time polymerase chain reaction (RT-PCR) was performed using the chosen primers and Quantitect Probe PCR Kit.

Results: One forward primer and two reverse primers were chosen for experimental optimization: F: 5’-CGATATCAGGGAAGATGTGC-3’, R1: 5’-TTAGGGGCAGCTACCTTAGC-3’ and R2: 5’-TCACAGGAATCGCACCCATT-3’. The probe sequence was CCAACATGGAAGGATATTCC. Both primer sets were able to successfully detect and replicate CAR-SIV mRNA from CHO-CAR cells. The threshold cycle value (Ct-value) for primer set with the first reverse (R1) primer was lower when compared with that of the second reverse primer (R2), indicating it to have a higher sensitivity in the tested reaction conditions. PCR product length was verified using gel electrophoresis.

Conclusion: Enterovirus infections are linked with autoimmune diseases such as T1D and celiac disease. One study reports a higher CAR expression among diabetes patients when compared with healthy individuals. More research is needed on how CAR regulates the viral effects. The PCR primers presented here can detect CAR-SIV isoform semi-quantitatively. A quantitative method can also be developed using these primers. The method presented here will be used in research evaluating the connection between CAR and T1D or celiac disease.

Keywords: Coxsackievirus and adenovirus receptor, CAR, CAR-SIV, Type 1 diabetes

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TIIVISTELMÄ

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Tausta: Coxsackievirus ja adenovirus reseptori (CAR) on proteiini, jolla on sekä solukalvon läpäiseviä että solunulkoiseen tilaan eritettäviä isoformeja. Coxsackie B -virukset sekä monet adenovirukset käyttävät CAR-molekyyliä reseptorinaan, minkä lisäksi CAR toimii polarisoituneiden epiteelisolujen välisissä tiiviissä liitoksissa solukalvot toisiinsa kiinnittävänä proteiinina. Polarisoiduissa soluissa eri isoformien sijainti solukalvolla poikkeaa toisistaan, mutta ei-polarisoiduissa soluissa tällaista eroa ei ole. Lisäksi haiman beetasoluissa CAR:n seitsemän eksonin koodaama CAR-SIV-isoformi sijaitsee insuliinin eritysrakkuloissa, eikä solukalvolla. Viime aikoina enterovirus-infektioilla, erityisesti Coxsackie B -virusinfektioilla, on havaittu yhteys autoimmuunisairauksiin, kuten tyypin 1 diabetekseen. CAR:n merkitystä tässä yhteydessä ei tiedetä, mutta Coxsackie B -virusten reseptorina se on mielenkiintoinen tutkimuskohde.

Työn tarkoitus: Työn tarkoituksena on selvittää, mitä CAR:n rakenteesta ja fysiologiasta, sekä yhteydestä tyypin 1 diabetekseen tiedetään, sekä millaisilla PCR-menetelmillä CAR:n ilmentymistä on tutkittu. Tämän pohjalta työssä suunnitellaan CAR-SIV-isoformin tarkasti tunnistava PCR-menetelmä, jota voidaan myöhemmin käyttää tyypin 1 diabetekseen ja keliakiaan liittyvissä tutkimuksissa.

Aineisto ja menetelmät: Kirjallisuushaku suoritettiin PubMed-tietokannassa. Kuusi sopivaa tutkimusta valittiin tarkempaan tarkasteluun, ja kaikki julkaistut PCR-alukkeet ja koettimet otettiin mukaan. Lopulta alkupään aluke- sekä koetinsekvensseiksi valittiin jo aiemmin julkaistut sekvenssit niiden sopivan sijainnin perusteella ja tämän lisäksi suunniteltiin kaksi uutta sekvenssiä CAR-SIV:n uniikille alueelle loppupään alukkeiksi. Menetelmän toimivuutta testattiin ihmisen CAR:lla transfektoiduissa hamsterin munasarjasolulinjan soluissa (CHO-CAR). Negatiivisina kontrolleina käytettiin CHO-soluja, joissa ei ole ihmisperäistä DNA:ta. RT-PCR suoritettiin valmistajan ohjeiden mukaan Quantitect Probe PCR Kit:llä. PCR-tuotteen pituus varmistettiin geelielektroforeesilla.

Tulokset: Menetelmässä käytettävien alukkeiden sekvenssit valittiin seuraavasti: F: 5’-CGATATCAGGGAAGATGTGC-3’, R1: 5’-TTAGGGGCAGCTACCTTAGC-3’ ja R2: 5’-TCACAG-GAATCGCACCCATT-3’. Koetinsekvenssi oli 5’-CCAACATGGAAGGATATTCC-3’. Molemmat alukeparit monistivat CAR:n RNA:ta CHO-CAR soluista. Ensimmäisen alukeparin (F ja R1) treshold cycle -arvo oli matalampi verrattuna toiseen pariin (F ja R2), joten tutkituissa reaktio-olosuhteissa ensimmäinen alukepari vaikutti olevan herkempi tunnistamaan CAR SIV:tä

Yhteenveto: Enterovirus-infektiot ovat yhteydessä autoimmuunisairauksiin, kuten tyypin 1 diabetekseen ja keliakiaan. Yhdessä tutkimuksessa on raportoitu CAR-ekspression olleen korkeampi diabetesta sairastavilla kuin terveillä verrokeilla. Tämän yhteyden patofysiologiset mekanismit ovat epäselviä. Tässä tutkimuksessa kehitettyä PCR-menetelmää voidaan käyttää CAR:n ja tyypin 1 diabeteksen sekä keliakian välisen yhteyden tutkimisessa.

Avainsanat: Coxsackievirus ja adenovirus reseptori, CAR, CAR-SIV, tyypin 1 diabetes

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LIST OF SYMBOLS AND ABBREVIATIONS

AdV5-Beta-Gal	Beta-galactosidase gene carrying recombinant adenovirus
CAR	Coxsackievirus and adenovirus receptor
CHO	Chinese hamster ovary
Ct-value	Treshold cycle value
CVB	Coxsackie B viruses
EC	Endothelial cells
EV	Enterovirus
F	Forward
MEC	Microvascular endothelial cells
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
R	Reverse
RT-PCR	Real time PCR
SNP	Single-nucleotide polymorphism
T1D	Type 1 diabetes
TEDDY	The Environmental Determinants of Diabetes in the Young study

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1 INTRODUCTION

The coxsackievirus and adenovirus receptor (CAR) is a protein first isolated in 1997 (1), after which it has become an important subject of scientific research in various areas of medicine. A multitude of studies have already been conducted, examining the connection between CAR and for example different cancer types, cardiac and neural physiology and type 1 diabetes (T1D) (2–8).

As its name implies, along with its many physiological functions, CAR also mediates the attachment and entry of coxsackie B viruses (CVB) and many adenoviruses into human cells. (1). Recent studies have suggested a possible connection between these types of enterovirus (EV) infections and type 1 diabetes (T1D). It is believed that EV infections can be linked with at least a portion of environmentally triggered T1D cases. (9,10) Thus, it also seems conceivable that potential differences in individuals' levels of CAR gene expression and in its genetic configuration could play a factor in the pathogenesis of T1D.

Along with T1D, CAR looks like an interesting research point regarding celiac disease (CD) and adenoviral oncolytic therapies. Recently, studies have started to point out a connection between a higher amount of EV infections before CD onset compared to controls of the same age (11,12). To my knowledge, no data is currently available on how, or if, CAR relates to CD. Adenoviral oncolytic therapies use adenoviral vectors for tumour specific treatments and possibly also genetic modification of the tumours to better respond to already established treatments (13). Understanding the functionality of CAR could prove useful in this regard as well, because many adenoviruses use CAR as a receptor for cell entry.

The interactions between viruses, an individual's immune system and variation in the genes that influence the course of infection are complex. Thus, the pathophysiological mechanics following EV-infection, possibly leading to onset of T1D, are especially interesting. Understanding these mechanisms further could make it possible to develop interventions and give treatments already before disease onset in high-risk individuals.

2 AIMS

The aims of this work are as follows: First, to review the current existing data on CAR, its composition, functions, and connections to different physiological subsystems and type 1 diabetes. Second, to identify previously used PCR protocols for CAR detection in mouse or human tissues. And third, to then design primers for a quantitative PCR (qPCR) assay, capable of detecting the seven-exon transmembrane isoform of CAR (CAR-SIV/CAR^{Ex7}) to be eventually further developed and used for the analysis of human samples.

3 THE COXSACKIEVIRUS AND ADENOVIRUS RECEPTOR

3.1 CAR isoforms

The coxsackievirus and adenovirus receptor is an Ig-like cell adhesion molecule encoded by the *CXADR* gene located in chromosome 21q21.1 (14). The gene is comprised of eight exons, the first five of which are responsible for encoding the extracellular part of the receptor. The transmembrane domain is located in the first half of exon six, and the remaining half of exon six along with exons seven and eight encode the intracellular part. This is significant, because the five different isoforms of CAR are spliced in different locations such that three isoforms are entirely extracellular, and thus are excreted out of cytoplasmic space. The two remaining isoforms, CAR-SIV and CAR-TVV include the transmembrane domain and correspondingly remain at the cell surface after translation. The SIV- and TVV-isoforms differ from each other only in the intracellular portion. (15,16) The genetic structure of *CXADR* and the different CAR isoforms are shown in more detail in Fig.1.

The functionality of these structurally distinct isoforms has been studied in many contexts, and the results remain somewhat uncertain. It is thought that due to their structure spanning over the cell membrane, CAR-SIV and CAR-TVV can mediate viral entry into cells, but the factors influencing and regulating the expression of these isoforms as well as their role in viral infections seems complex. (17) The soluble extracellular isoforms have been shown to bind with both CAR-SIV and CAR-TVV, as well as with coxsackie B viruses, meaning it is probable that they could also modulate the course of infection, either by blocking or enhancing viral infectivity, or alternatively, contributing to the host's immune response.

The soluble isoforms, however, have also been shown to differ significantly in this regard. Coxsackievirus B3 has been shown to bind to isoform CAR4/7 but not to isoform CAR2/7 (16). The effect of CAR4/7 treatment in adenoviral myocarditis was examined in mice, with complicated results. Daily CAR4/7 treatment led to decreased viral load, but was also associated with significantly increased myocardial inflammation and tissue damage (18). It was determined that the effect was not only caused by the addition of CAR4/7, because non-infected mice treated similarly did not develop such tissue damage within the study's timeframe. Thus, more research is needed on the fundamental functionality and interactions of all the CAR isoforms.

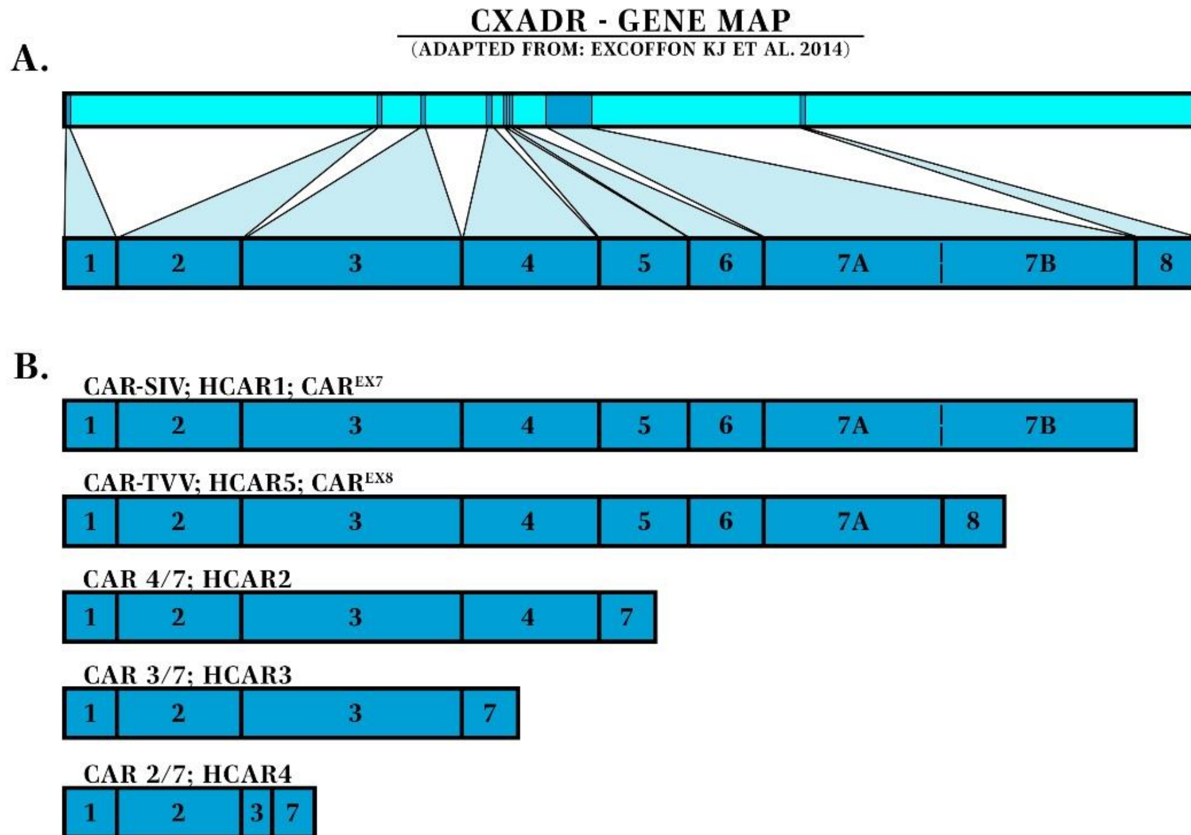

Figure 1. A: The exon structure and localisation of the CXADR gene visualized. B: Differences in mRNA splicing between the CAR isoforms is depicted. Note that several names have been used for each of the isoforms in previous literature. (Adapted from Excoffon et al. 2014)

3.2 CAR tissue localisation and functionality

As previously mentioned, CAR functions as a cell-adhesion molecule in its physiological role. The two transmembrane isoforms seem to also differ in their cellular localisation, shown in polarized epithelial cells of human airways. CAR-TVV seems to be more prevalent on the apical surface, while the majority of CAR-SIV localizes to the basolateral parts of the cells. (19) The factors influencing these differences are not yet understood, and could be interesting to examine, because the extracellular and transmembrane domains are structurally identical. This suggests that maybe some feature of the intracellular domain functions in determining the molecule's location and functionality. Another study examined the properties of different types of extracellular domain deletion mutants and found similar results. The extracellular domain is not needed for the molecule to be expressed and located on the cell membrane. However, mutated receptors were unable to stay

localized on the basolateral membrane. Thus, correctly functioning intracellular and extracellular domains are required for CAR-mediated cell-to-cell junctions and adhesion to function properly. (20)

In addition, CAR appears to be critical in the early embryonal development of cardiac tissue in mice. When the start codon of murine CAR gene is deleted, the resultant homozygous mutant embryos are unable survive, resulting in death by day 13.5 of embryonal development. (21) Furthermore, several studies have been performed regarding CAR and embryonal development: CAR interacts with extracellular matrix glycoproteins and is involved with neurite extension in the developing nervous system (3). Irreversible morphological changes are also seen in mice with tamoxifen-inducible CAR knockout genes. The intestines appear dilated and notable atrophy is seen in the pancreas. (22) Similarly performed inducible CAR knockout was observed to also lead to abnormal and dilated lymphatic vessels in CAR-deficient embryos (23).

CAR is shown to be expressed widely across the human body. Tissue types which are believed to strongly express CAR on the protein level include the lungs, stomach, duodenum, gallbladder, kidneys, urinary bladder and skin. (24) This seems logical since these tissue types include epithelial cells, and cell-to-cell adhesion is crucial for the integrity of these tissues to form a barrier between underlying tissue and outside air or between different body cavities.

A more recent study examined the expression and localization of CAR in the human pancreas (25). A high level of protein expression was observed in the case of CAR-SIV in pancreatic beta cells, which locate to endocrine pancreas i.e., the pancreatic islets. In the exocrine pancreas CAR expression was clearly lower. CAR-TVV was also present in beta cells, but at a lower level. A surprising finding is that CAR-SIV was shown to localize in the cytoplasm, rather than on the cell membrane as previously presented. Further examination indicated CAR-SIV to co-localize with proteins found in insulin secretory granules. A hypothesis can be derived from these findings, suggesting that the act of insulin secretion at the cell membrane of beta cells enables the CAR-SIV extracellular domain to be exposed into extracellular space. This opens a possible gateway for the virus into beta cells.

3.3 Enterovirus, CAR and type 1 diabetes

The etiology of T1D still remains unclear despite decades of research both before and after the first insulin treatments authorized for use in humans in 1922 (26). The discovery of this life-saving treatment has enabled many patients to be able to evade an otherwise imminent untimely death. In the last years, we have seen a constant and clear increase in the incidence rate of the disease globally, which yields additional importance for both the treatment of T1D and the possible

preventative measures, which we are yet to find. (27) Nowadays it is widely accepted that the etiology of T1D is multifactorial by nature.

As stated earlier, previous studies have shown a significant connection between EV-infections and T1D. A meta-analysis, which included 25 studies, presented EV-infections to be significantly related to clinical T1D with a combined OR of 5.75 (95% confidence interval (CI) 3.61 – 9.16) (28). A limitation of this meta-analysis is the fact that the individual studies included were somewhat heterogenic by nature, and due to their retrospective setting, all confounding factors can't be considered when interpreting the results. Nevertheless, a significant connection between EV-infections and T1D was observed, and it is perhaps possible, that some of the differences among the individual studies' results could be also explained with the regional differences in other T1D risk factors, such as genetical susceptibility. Another larger meta-analysis with 5921 subjects from 38 studies also reported a significant association between enteroviral infections and T1D in European, African, Asian, Australian and Latin American populations, but not in North America (29).

EVs have been shown in the blood, gut and pancreas tissue samples of patients with T1D. These findings are also proportionately more frequent when compared with those of healthy individuals. (9,30) The DiViD study examining pancreatic tissue samples from six live adult patients in Norway showed a significant pattern of antiviral response in all six patients' samples. This increase was markedly different when compared to non-diabetic controls. (31) In addition, all six samples were positive for EV genomes, detected with immunohistochemistry and RT-PCR. Out of eleven adenocarcinoma cases examined similarly, only two were found positive for EV-RNA. Notably, only one diabetes case and one control case showed positivity for other viruses, further indicating EV-infections to be of special importance in this regard. (32)

CAR presents itself as an interesting study point in this process, because as indicated earlier, many EVs bind to it, and it has been shown to be expressed in the pancreas of individuals with T1D. In the previously mentioned DiViD study population, CAR-expression was shown to be higher in samples from individuals with T1D, or at a higher risk of T1D, in comparison with individuals without T1D. (7) An earlier study examined the expression of CAR in pancreatic islet microvascular endothelial cells (MEC) and aortic endothelial cells (EC), finding CAR to be expressed in both. However, when these cell lines were infected with CVB, an increase in expression was seen in the pancreatic islet MECs, whereas in the aortic ECs CAR expression decreased. (33)

Thus, the process of viral infection into cells via CAR seems more complex than just depending on the receptor expression. Expression of CAR has been examined in multiple different cell lines. When comparing non-polarized and polarized cells infected with beta-galactosidase (AdV5-Beta-Gal) gene carrying recombinant adenovirus, a significant difference was seen. In non-polarized

cells the activity of beta-galactosidase was significantly higher across all cell lines compared with that of polarized cells. This shows viral activity to have been higher in non-polarized cells despite the overall CAR expression having been quite similar between the cell lines, except for a few isoform-specific differences. (17) Note, that in this study CAR expression was not examined after virus introduction so it is not known whether the viral infection further affected the levels of CAR. Another experiment with recombinant doxycycline inducible CAR-TVV showed a decrease in adenoviral infection when the eighth exon was inactivated in polarized cells. The effect was reversible with adenoviral infection increasing after induction of CAR-TVV. (34) This indicates the CAR-TVV isoform to be especially important in luminal adenovirus infection.

Also, in The Environmental Determinants of Diabetes in the Young (TEDDY) study, it was found that *CXADR* rs6517774, a single-nucleotide polymorphism (SNP) in the *CXADR* gene, was associated with pancreatic islet autoimmunity (8). The significance of this, and perhaps other genetic differences in *CXADR* and other genes related to EV infections and the immune reaction which follows, is also yet to be fully understood. Another finding in the TEDDY study was that a persistent EV-B infection was more related with islet autoimmunity than shorter, more acute infections (8). These persisting infections are an interesting new target for T1D research, because we don’t yet fully understand the effects they might have on the infected cells and possibly the immune system’s response against them.

4 MATERIALS AND METHODS

4.1 Selecting PCR-primers and probes for human CAR

The first aim of the study included a literature search and comparison of previously published real time PCR (RT-PCR) methods used for the amplification of CAR specific mRNA and its different isoforms in human cells. The search was performed on PubMed.

Based on literature the aim was to choose a probe sequence and design a set of primers for a PCR-protocol capable of selectively detecting the mRNA of the CAR-SIV isoform, which may play an important role in the human beta cells of the pancreas (25).

The primer set design was followed by examination of the primers' properties and location and suitability of the primers for CAR-SIV detection. These steps are described in detail in the results section. The overview of the steps of this study is described in Fig.2.

4.2 Cell lines

CHO cell line was used to validate the amplification of CAR gene using RT-PCR. In addition to native CHO cells which do not express CAR, a stable CHO transfectants expressing human CAR were obtained from prof. Magnus Lindberg (Kalmar University, Kalmar, Sweden). Cells were cultivated as described previously (35).

4.3 RNA extraction, cDNA synthesis and RT-PCR

RNeasy Mini kit, manufactured by Qiagen, was used according to manufacturer's instructions to obtain RNA from CHO cells. Cell lysate was homogenized using QIAshredder. Promega M-MLV Reverse Transcriptase was used for cDNA synthesis. Quantitech Probe PCR kit was used for cDNA amplification. All of the mentioned steps are presented in more detail in the results section of this report.

In addition, qPCR of a housekeeping gene 18SrRNA (36) using Thermo Fisher Scientific's DyNAmo Flash SYBR Green qPCR Kit was performed to ensure that the RNA extraction and cDNA synthesis were performed successfully.

RNA extraction from three samples each of CAR positive CHO-CAR cells and CAR negative control cells was performed using the RNeasy Mini kit and QIAshredder (Qiagen). 600 µl of buffer RLT was added to each sample to achieve cell lysis. The lysate was pipetted into QIAshredder

spin columns placed in 2 ml collection tubes and sentrifuged for 2 minutes at full speed to homogenize the samples. After homogenization, 600 µl of 70 % ethanol was added to each of the samples and mixed by pipetting. From these mixtures, 600 µl was transferred onto RNeasy spin columns and centrifuged for 15 seconds at >8000 g, after which the flow-through was discarded. This step was performed twice for each sample due to the total volume being approximately 1200 µl. Next, 700 µl of RW1 buffer was added to each of the spin colums and centrifuged similarly for 15 seconds at 8000 g. Flow-through once again discarded. Following this, 500 µl of RPE buffer was added to each of the spin columns and centrifuged once more for 15 seconds at 8000 g, discarding the flow-through. And lastly, a second 500 µl of the RPE buffer was added onto each spin column and centrifuged for 2 minutes at 8000 g to finish the washing of the spin column membrane. Flow-through was discarded once more and the remaining RNA was eluted into 40 µl of RNAse free water and centrifuged for 1 minute at >8000 g to collect the final RNA samples.

The 6 RNA samples were converted into cDNA with Promega M-MLV (Moloney Murine Leukemia Virus) reverse-transcriptase (RT) enzyme and random hexamers. First, 20 µl of each of the samples was combined with 2,8 µl of random hexamers, mixed and spun, and incubated at 70 °C for 5 minutes. Meanwhile, a RT master mix for our six samples was made using 70 µl of Promega 5x reaction buffer, 70 µl of 2.5 mM dNTP, 8,4 µl of RNAse inhibitor, 28 µl of nuclease free water and 14 µl of the M-MLV enzyme. After the first incubation, the tubes were placed on ice and cooled immediately, followed by the addition of 27,2 µl of the RT master mix. Each reaction mixture was then mixed thoroughly and centrifuged briefly to avoid specimen remaining on the sides of the tube. For the first strand synthesis and extension, the tubes were then incubated at 37 °C for 60 minutes. CDNA was stored at -20 °C after synthesis.

To verify that the RNA extraction and cDNA synthesis were performed successfully, qPCR of a housekeeping gene 18SrRNA was first performed (36) using Thermo Fisher Scientific's DyNAmo Flash SYBR Green qPCR Kit. For each reaction mixture, 12,5 µl of SYBR Green DynamoFlash master mix was combined with 1,25 µl of 10 µM forward and reverse primers for 18SrRNA, 0,5 µl of ROX dye and 7,25 µl of water. 2,5 µl of template cDNA and 22,5 µl of reaction mixture constituted a reaction volume of 25 µl. A negative control was made with same reagents excluding any template to minimize the chances of false results. The thermal cycler conditions were programmed with first initialization at 95 °C for 7 minutes, denaturation at 95 °C for 10 seconds and combined

annealing and extension at 60 °C for 30 seconds. A total of 40 cycles were performed with final extension at 60 °C for 60 seconds. PCR product was stored at 4 °C immediately after multiplication.

Finally, Quantitect Probe PCR Kit (Qiaqen) was used according to manufacturer's instructions in our CAR-SIV PCR protocol. Primers were diluted to 10 µM and the probe to 25 µM prior to their use. For each reaction mixture 5 µl of 2x Quantitect probe PCR master mix was combined with 0,3 µl of the forward and reverse CAR primers, 0,08 µl of the CAR probe, 1,72 µl of RNAse free water and 2 µl of the cDNA template. The plate was sealed, the mixtures mixed thoroughly, and the plate finally centrifuged briefly to avoid mixture residue on the sides of the wells. The thermal cycler conditions were programmed with first initialization at 95 °C for 15 minutes, denaturation at 94 °C for 15 seconds and combined annealing and extension at 60 °C for 1 minute. A total of 50 cycles was performed with data collection done every cycle after annealing and extension. Both primer sets were tested on the same plate in the same reaction conditions. PCR product was stored at 4 °C immediately after multiplication. The reaction was performed using Applied Biosystems' 7500 Real-Time PCR System running 7500 System SDS Software.

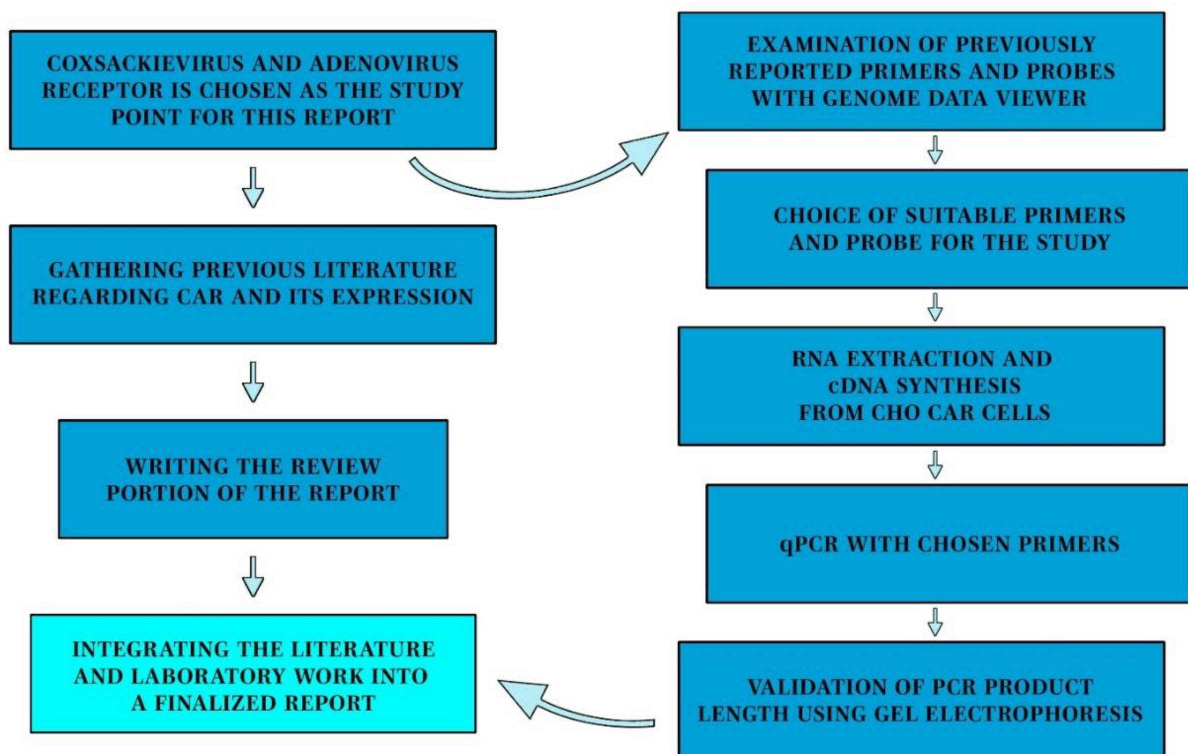

Figure 2. A flow chart visualizing each of the steps of the study.

5 RESULTS

5.1 PCR-primers for human CAR in previous literature

My first aim was to find and compare previously published RT-PCR methods used for the amplification of CAR specific mRNA and its different isoforms in human cells. A search was performed on PubMed, and in the end, six publications with possibly suitable PCR primers were chosen to compare. The details of each of the provided primers and probes are presented in Table 1.

5.2 Primer & probe design

The second aim of this study was to attempt designing a set of primers for a PCR-protocol capable of selectively detecting the mRNA of the CAR-SIV isoform. This isoform was selected based on previous literature indicating it to be of special importance in human beta cells (25).

In order to choose our primers for CAR-SIV, The National Library of Medicine's Genome Data Viewer (37) was used to examine the location and suitability of the primers and probes presented earlier. A suitable forward primer (F) sequence should span over the splicing site between exons six and seven, which should allow the primer to bind only to the spliced mRNA product and not genomic DNA. The CAR-SIV probe sequence used in the Ifie et al. (25) study met this criteria and thus was chosen as our forward primer sequence. A suitable reverse primer sequence should be located on exon seven on the part specific to CAR-SIV, located after the intraexonic splice site for CAR-TVV. Two previously unpublished, slightly differing reverse primers (R1, R2) were chosen to compare their results.

The probe sequence was also chosen based on previous literature (25), located in the first part of exon seven between the primer pairs' bonding sites. Northwestern University's OligoCalc (38) was used to check the primers' properties to minimize the possibility of self-dimerization and hairpin formation. The chosen primers and probe are presented in Table 2 and their genetic localization and predicted PCR product lengths are visualized in Fig.3.

Author	PCR type	Primer sequences 5'-NNN-3' (if available)	Probe sequences 5'-NNN-3' (if available)
Sharma et al. (2017)	qPCR (cDNA)	F: TCGGCAGTAATCATTCATCCCTGG R (SIV): ATAGACCCATCCTTGCTCTGTGCT R (TVV): ACTGTAATTCCATCAGTCTTGTAAGGG	Not specified
Ifie et al. (2018)	RT-PCR (RNA)	F: GGAAGTTCATCACGATATCAG R (SIV): AATCATCACAGGAATCGCAC R (TVV): TTCCATCAGTCTTGTAAGGG	Not specified
Ifie et al. (2018)	qRT-PCR (RNA)	Not specified	SIV: CGATATCAGGGAAGATGTGC TVV: CCAACATGGAAGGATATTCC 2/7: GGTGGATCAAGTGGGAAGAT 3/7: GTAGTTCTTGGGAAGATGTG 4/7: CATGGTTAGCAGGGAAGATGT
Excoffon et al. (2009)	qRT-PCR (RNA)	F (SIV): TGCCAGAAGCTACATCGGCAGTAA R (SIV): ATAGACCCATCCTTGCTCTGTGCT F (TVV): AGGGAAGATGTGCCACCTCCAAA R (TVV): CAACTGTAATTCCATCAGTCTTGTAAG	SIV: AAGTCGAATGGGTGCGATT-CCTGTGA TVV: ACTGCCAGAAGCTA-CATCGGCAGTAA
Hodik et al. (2016)	RT-PCR (cDNA)	Not specified	Not specified
Zanone et al. (2007)	RT-PCR (cDNA)	F: GACTCA-CAGAAAATGCCCAC R: CGACAGCAAAAGATGATA-AGACC	Not specified
Tatrai et al. (2011)	qPCR (cDNA)	EXON1 F: CTGGTTTAGCTTAAGGGAT / R: CCTACTCACCCACTACTC EXON2 F: GTATCCCTCGCATCAATG / R: GCGAGCAAGGACATATCA EXON3 F: GTGTGTTTGTTTTTCCTCCT / R: ACAAGTGCTGCTACTACA EXON4 F: AACCCAGAACCAACTGAT / R: TCACACACACACCCCTACTA EXON5 F: CTTTTTCCTCCTTCCATAGA / R: GAAACAAAACCACCAACAG EXON6 F: TAGCCTACCTTCAGTATC / R: TCCTGTCTCACTTAATTACC EXON7 F: CATGTATTGGGGATTTTGC / R: AAGGAAAGGAACACGGAGA	Not specified

Table 1: Primer and probe sequences presented in literature for CAR multiplication.

Primer	Sequences (5'-NNN-3')
Forward, F	CGATATCAGGGAAGATGTGC
Reverse 1, R1	TTAGGGGCAGCTACCTTAGC
Reverse 2, R2	TCACAGGAATCGCACCCATT
Probe	CCAACATGGAAGGATATTCC

Table 2: The final sequences chosen for use in this study.

Figure 3: Genetic localization of the primers and probe visualized. Note the forward primer's location on the splice site between exons 6 and 7. Predicted PCR product lengths are also presented for both primer sets.

5.3 Examination of CAR-SIV expression

The ability of the selected primer set/probe to detect CAR-SIV was tested using hamster CHO cells transfected with human CAR. Non-transfected CHO cells were used as negative controls and an additional negative control was made with only the PCR reagents excluding any template. The RT-PCR reaction was carried out as presented in section 4.3 of this report.

The results for all six samples were consistent, indicating that the primer sets can detect CAR-SIV mRNA. No product was detected from the negative controls (samples 1-3), while both primer sets yielded a positive result from the CHO CAR cells (samples 4-6). The cycle threshold (Ct-value) indicates the amount of reaction cycles needed for a template's fluorescence to rise above a set threshold level in relation with the background fluorescence of the reaction (ΔRn). This threshold should be set so that only the reactions in exponential or linear multiplication phase are able to cross it. (39) The results and Ct-values obtained from the PCR runs are summarised in Table 3.

Primers	Sample	CAR	Ct-value
F + R1	1	neg.	Undetermined
	2	neg.	Undetermined
	3	neg.	Undetermined
	4	pos.	19.093
	5	pos.	18.756
	6	pos.	18.891
	Control	neg.	Undetermined
F + R2	1	neg.	Undetermined
	2	neg.	Undetermined
	3	neg.	Undetermined
	4	pos.	29.787
	5	pos.	24.528
	6	pos.	27.341
	Control	neg.	Undetermined

Table 3: The Ct-values obtained with a treshold ΔRn of 1.6695e-003. Samples 1-3 are from CAR-negative CHO cells, and 4-5 are from CHO-CAR cells. Controls were made with PCR reagents and the template volume replaced with water.

5.4 Validation of PCR results

To further validate our results, The PCR product was examined using gel electrophoresis. 2 grams of agarose powder and 10 µl of Gelred stain was added into 100 ml of 1x TAE buffer and the mixture was heated in the microwave in short bursts and mixed until the agarose was completely dissolved. The mixture was allowed to cool slightly, before being poured into the appropriate mold and let cool completely. Meanwhile, 2 µl of loading dye was added to each sample and mixed thoroughly. After the gel had set completely, it was placed into a buffer tank filled with 1x TAE buffer. 100 bp ladder was pipetted into the outermost wells and the samples into the wells in between. The electrophoresis was run at 100 V for 60 minutes. The results are presented in Fig.4. As predicted by the PCR product lengths, the bands formed near the 200 bp ladder fragment, with F + R1 product being slightly shorter than F + R2 and thus migrating a longer distance. The gel image is presented in fig. 4.

The bands from F + R1 appear more uniform and brighter than those from F + R2. This, in addition to the Ct values presented earlier, suggests, that out of these two primer pairs, F + R1 yields a higher sensitivity when compared with F + R2 in these reaction conditions.

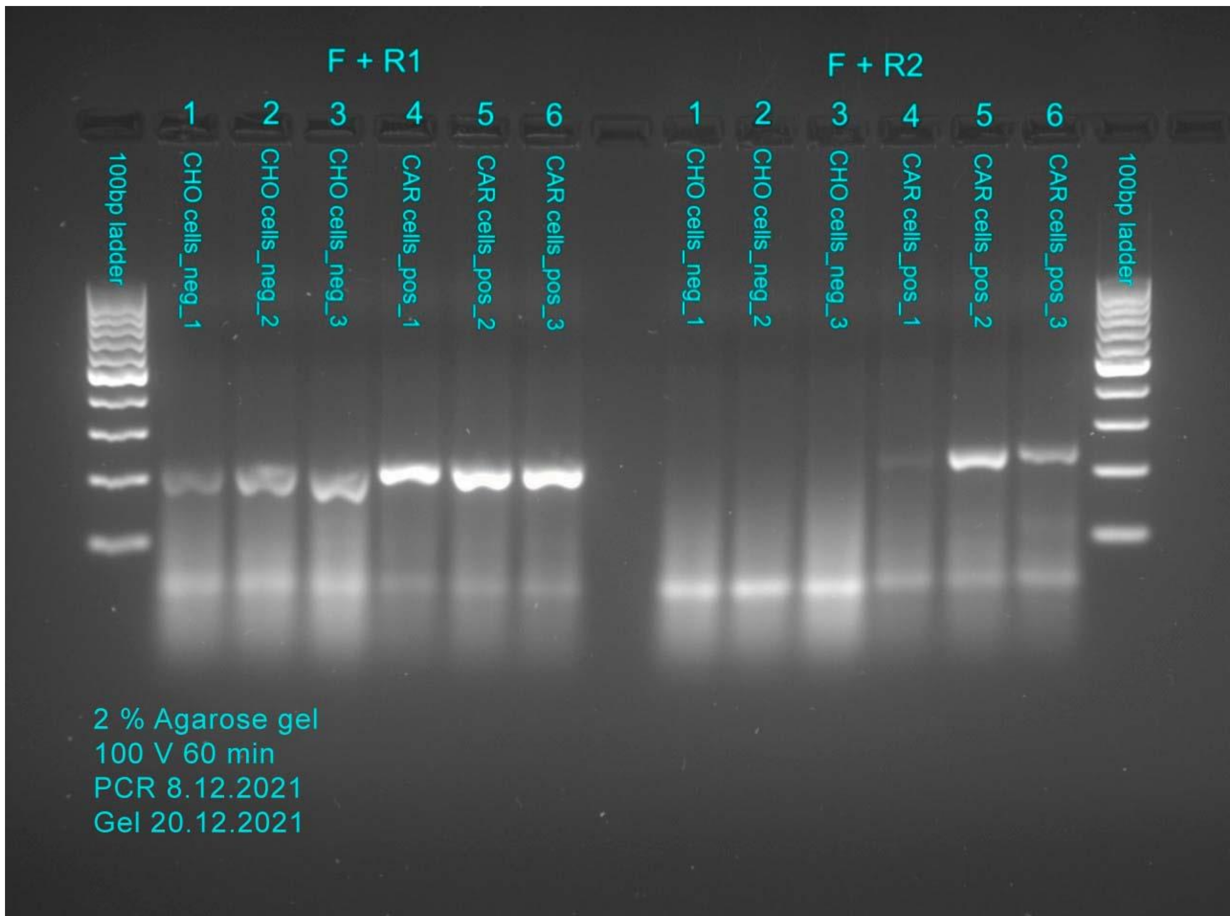

Figure 4: Image of the earlier obtained PCR product on 2 % agarose gel ran at 100 V for 60 minutes. Results are consistent with the product length predicted *in silico*.

6 DISCUSSION

Based on the currently available data, the coxsackievirus and adenovirus receptor, CAR, presents as an interesting research topic when it comes to T1D and many other diseases. Viral infections and their connection to different illnesses is a rapidly growing field of research, and thus CAR, as a receptor to a few viruses linked to, for example T1D, could play a significant role in the pathogenesis of these diseases. As of yet, there are still many unanswered questions regarding this topic and the mechanisms with which these diseases develop.

The structure of the CAR gene and its many isoforms is already well known and documented. The functionality and purpose of these isoforms, however, is not. As we presented here, the viruses which use CAR as a receptor, have been linked to T1D (28,29), but the role of CAR in this link is still somewhat unclear. In addition, the fundamental mechanisms behind islet cell autoimmunity and destruction are also mostly unknown. Several high-risk genes have been found and can be screened for in early childhood, but, in the end, all of this population is not affected. Why some high-risk individuals never contract the disease, and some with fewer risk factors end up affected is not known. The deciding factor, or indeed multiple factors, of disease onset has not been determined and therefore more research is needed. As presented earlier, the localization of CAR-SIV in the insulin secretory granules and the possible connection between viral infections and T1D is a promising direction for more studies. Another addition to this is the indication towards persistent infections being more potent in triggering the disease (8). More research is needed on how persistent infections change the pancreatic and other types of cells, and whether CAR plays a factor in this development. The ways in which the different CAR isoforms affect viral entry into cells and other physiological functions are interesting regarding this topic and also many others.

Another area of research where more information on CAR is needed is oncolytic virus therapy, such as adenoviral therapies for cancer. CAR has been shown to be upregulated by some cancer types and downregulated by some. What this means for the pathology of these cancers is not known. (40) For adenoviral therapies to have a positive effect, we have to be able to target them specifically against the cancer cells without affecting healthy cells. CAR and other adenoviral receptors play a major role in this regard, both in the first infection into cancer cells and possibly in cell-to-cell lateral spreading. For a treatment to be effective, it must reach even cells which are not immediately in contact with blood vessels or other luminal spaces where therapies are able to be administered. The differing cellular localisation of CAR isoforms could facilitate this kind of spread within more solid cancer tissue and thus increase the efficacy of the treatments. In order to achieve

this, however, we must also gain more knowledge on the physiological expression and role of CAR in healthy tissue, to avoid targeting it with cancer treatments. Also, therapies modulating the CAR expression of healthy and cancer tissues independently of one another, could increase both the specificity and effectiveness of viral therapies.

As of yet, to our knowledge, there are no studies performed on the specific regulation of different CAR isoforms and their expression. To gain information on this, more ways of studying CAR and its isoforms on the genomic and proteomic level are needed. In this report the aim was to investigate the previously used PCR methods in the analysis of CAR. I was able to identify multiple different studies with a variety of primers used to achieve this. Out of these sequences a few suitable ones were chosen, and the reverse primers were designed as best fit for our purpose. In its current state, the primers and PCR protocol used can qualitatively and semi-quantitatively detect the CAR-SIV isoform and produce a PCR product with the length predicted *in silico*.

To achieve quantification, a few options are available. The standard curve method would involve more PCR runs with sequential dilutions of the template in order to generate a standard curve, from which the theoretical geometric efficiency could be calculated and used along with the Ct values to achieve a baseline quantity. Another option is the ΔΔCt method, where the obtained Ct values are normalized to a normalizer gene and calibrator sample, and the quantity can be calculated using the $2^{-\Delta\Delta Ct}$ equation. (41) These steps were not yet completed in this report, due to time and sample sufficiency reasons. This is currently the biggest limitation of this study, however, the steps mentioned above can be carried out later and thus a quantitative method can be achieved. Another limitation is the fact that CAR mRNA or protein expression was not absolutely verified with another specific method. To combat this, certain steps were taken in the primer design process to minimize the chance of non-specific product formation. The primers were checked for self-dimerization and hairpin formation using OligoCalc (38). Also, the primer sequences and their complementary sequences were searched on Genome Data Viewer (37) and it was deemed unlikely for other product to be able to form. This, along with the forward primer's location on the exon splice site and the correct product length indicated by gel electrophoresis suggest a high specificity to CAR-SIV mRNA for our primers. Thus, despite some of the current limitations, the presented method has a few clear advantages for semi-quantitative, or quantitative examination of CAR-SIV expression, and shouldn't be able to multiply human genomic DNA.

This presented method is planned to be used in the future by the Tampere University Virology group in research regarding the connection between EV infections and T1D, and possibly celiac disease (CD). CAR-SIV expression can be compared between tissue samples from T1D patients and healthy controls, and the earlier mentioned connection between *CXADR* SNP rs6517774 and T1D could be examined on the gene expression level. In addition, from a human pancreatic beta

cell line (EndoC) it would be possible to examine how other genetic modifications of the *CXADR* gene affect EV infectivity and beta cell functionality. Along with T1D, recent studies have pointed out a possible connection between CD and an elevated number of EV infections prior to disease onset (11). It is already well documented, that T1D and CD share many common risk factors and often coexist with one another (42). The pathophysiologic mechanisms behind this link and both diseases' onset, however, still need more research. Yet, no definitive answer on how CAR expression affects T1D and other diseases, or vice versa, is available. The method presented in this study provides an additional means for further elucidating the possible role of CAR in the pathogenesis of T1D and CD.

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