**Research Article**

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# Expression of sialic acid binding receptors (siglecs) in human trophoblast cell line

## *İnsan trofoblast hücre hattinda sialik asit bağlayici reseptörlerin (siglecs) ekspresyonu*

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**Abstract**

**Purpose:** Cell surface proteins known as Siglecs exhibit a specific affinity for sialic acid. Primarily located on the surface of immune cells, Siglecs belong to the subset of lectins called I-type lectins. Siglecs have important roles in maternal-fetal immune tolerance. We aimed to analyze the expression of Siglecs on Human Villous Trophoblasts (HVT) cells.

**Materials and methods:** Total RNA was extracted from the HVT cell line, cDNA was synthesized, and real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed the determine the changes in Siglec -3, -5, -6, -7, -8, -9, -10, -11, and -16 mRNA levels. In addition, Siglec levels were assessed by using immunohistochemical staining. Immunoreactivity against Siglec-6 and Siglec-9 was evaluated separately according to the intensity of brown color.

**Results:** Expression levels of Siglec genes by qRT-PCR and melting curve analyses were performed using RNA extracted from the HVT cell line. Siglec -3, -5, -6, -7, -9, -10, -11, and -16 genes were found to be expressed in the HVT cell line. Differently, Siglec-8 results were undetected after cycle 40, which was considered a negative result. The immunocytochemical examination of the HVT cell line revealed that Siglec-6 expression was moderate in the cytoplasm (Score:2). Siglec-9 expression was prominent in the cytoplasm (Score:3).

**Conclusion:** The results showed that Siglec-6 and Siglec-9 were expressed more than other Siglec proteins in the human trophoblast cells. Immunocytochemistry results also support these findings. Our study is the first to show Siglec-9 expression in human trophoblast cells.

**Keywords:** Siglecs, human villous trophoblasts, sialic acid.

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**Öz**

**Amaç:** Siglecler olarak bilinen hücre yüzeyi proteinleri, sialik asit için özel bir afinite sergilemektedir. Öncelikle bağışıklık hücrelerinin yüzeyinde bulunan Siglec'ler, I-tipi lektinler adı verilen lektin alt kümesine aittir. Siglecler maternal-fetal immün toleransta önemli rollere sahiptir. Bu çalışmada İnsan Villöz Trofoblast (HVT) hücre hatlarında Siglec tiplerinin belirlenmesi amaçlanmıştır.

**Gereç ve yöntem:** HVT hücre hattından total RNA ekstrakte edildi, cDNA sentezlendi ve Siglec -3, -5, -6, -7, -8, -9, -10, -11 ve -16 gen ekspresyonu Gerçek Zamanlı Kantitatif Ters Transkripsiyon Polimeraz Zincir Reaksiyonu (qRT-PCR) ile analiz edildi. Ek olarak, Siglec seviyelerini belirlemek için immünohistokimyasal boyamalar yapılmıştır. Siglec-6 ve Siglec-9' a karşı immünoreaktivite kahverengi rengin yoğunluğuna göre ayrı ayrı değerlendirilmiştir.

**Bulgular:** HVT hücre hattından ekstrakte edilen RNA kullanılarak Siglec genlerinin qRT-PCR ile ekspresyon seviyeleri ile erime eğrisi analizleri yapıldı. Siglec -3, -5, -6, -7, -9, -10, -11 ve -16 genlerinin HVT hücre hattında ifade edildiği bulundu. Farklı olarak, Siglec-8 sonuçları 40. döngüden sonra tespit edilemedi ve bu da negatif bir sonuç olarak kabul edildi. HVT hücre hattının immünositokimyasal incelemesinde Siglec-6 ekspresyonunun sitoplazmada orta düzeyde olduğu ortaya çıkmıştır (Skor:2). Siglec-9 ekspresyonu sitoplazmada belirgindi (Skor:3).

**Sonuç:** Sonuçlar Siglec-6 ve Siglec-9'un HVT hücre hattında diğer Siglec proteinlerinden daha fazla ifade edildiğini göstermiştir. İmmünositokimya sonuçları da bu bulguları desteklemektedir. Çalışmamız insan trofoblast hücrelerinde Siglec-9 ekspresyonunu gösteren ilk çalışmadır.

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**Anahtar kelimeler:** Siglecs, insan villöz trafoblast, sialik asit.

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## Introduction

Infertility is the absence of conception following one year of unprotected and regular sexual intercourse [1]. The causes of infertility can be categorized simply as female only, male only, both male and female and unexplained causes. However, infertility is a complex, multifactorial condition. Therefore a more detailed evaluation for a definitive diagnosis is important to determine the most appropriate treatment for couples [2-4]. With the development of new treatment protocols in ovulation induction, the success rates in infertility treatment with assisted reproductive techniques have increased, considering the high rate of high-quality oocytes obtained and advances in fertilization and embryo development. Nevertheless, the desired level of embryo implantation, especially after high-quality embryo transfer, has not yet been achieved [5]. Although several parameters have been proposed to determine endometrial receptivity, no positive results have been obtained for clinical use [6]. Endometrial factors that increase endometrial receptivity and improve implantation rate may be new therapeutic targets [7].

Sialic acids are nine-carbon sugars synthesized in vertebrates. They are the last sugars complementing various glycosylation structures [8]. Some bacteria have developed to synthesize “Sialic acids mimic”, de-novo synthesize, and take up them or sialyl-coated structures from the host to provide a survival advantage. Cancer cells use a similar strategy to evade the immune system. There is extensive immunological suppression through sialylated mucins produced by the tumor. Indeed, sialic acids associated with abnormal glycosylation and increased sialylation have been demonstrated in many tumors, such as colon cancer, renal cell carcinoma, prostate cancer, head and neck squamous cell carcinoma, breast cancer, oral cancer, and have been shown in the progression and metastasis of cancer [9-14]. The primary role of sialic acids in the immune system is thought to be due to the fact that they contain ligands for Selectin or calcium-dependent *C-type* leukocyte motility regulating lectins [8]. Activation of immune cells seems to be related to decrease sialic acids in cell surface [15]. Sialic acids are ligands for cell adhesion molecules in the sialic acid binding immunoglobulin-like lectins (Siglecs) family, which regulate the immune response [8]. Monocytes, macrophages, and dendritic cells contain largely the same Siglec profile. Synthesis of *Siglec-3, -7, -9* are high and synthesis of *Siglec-1* and *Siglec-10* are low after stimulation with IFN-α [16-23]. An intracellular “immunoreceptor tyrosine-based inhibition motif (ITIM)” is present in most Siglecs. ITIM stimulates inhibitory signal when it binds Sialic acids [24]. *Siglec-5* to *Siglec-11* carrying ITIM in their cytoplasma are called inhibitory Siglecs [25]. In contrast, *Siglec-4* and *Siglec-16* do not contain an ITIM. Therefore they are called activating Siglecs. *Siglec-14* may be an inducer of an inflammatory response through activation of the MAPK pathway [26]. Siglecs are ligands expressed primarily on macrophages and dendritic cells that are involved in cell adhesion and the internalisation of sialic acid-expressing pathogens. In innate cells, most Siglec-associated ITIMs are thought to protect against the development of autoimmunity by attenuating inflammatory responses in various cell types, mainly through their cis-interacting with sialoglycoconjugates. However, pathogens and tumors can subvert these inhibitory responses for their own purposes. While immune stimulation is important for the treatment process in cancers, the overactive immune system in allergies and autoimmune diseases needs to be suppressed in some way [27].

It has been suggested that Sialic acids and Siglecs have important roles in maternal-fetal immune tolerance and, therefore, may have positive or negative effects on the implantation success of the embryo, depending on whether this system is functioning properly or not. In this context, it is essential to demonstrate

the presence of Siglecs in trophoblast cells. Evaluating the Siglec relationship may be clinically significant in women diagnosed with infertility, recurrent pregnancy loss, and recurrent implantation failure in assisted reproductive techniques treatments. In this study, we aimed to determine Siglec types in trophoblast cells.

## Material and methods

### Cell culture

Human Villous Trophoblasts (HVT) (Cat#910958, Innoprot) cell lines were obtained Cells cultured in DMEM/F12 (Sigma) supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO), 1% penicillin/streptomycin (GİBCO) mix and were incubated at 37°C with 5% $CO_2$ [28].

### Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the innuPREP RNA Mini Kit 2.0 (Analytik Jena, Germany) according to the manufacturer's protocol. A total of 2.5 µg RNA was reverted to cDNA by using an A.B.T.™ cDNA Synthesis Kit according to the manufacturer's instructions. qRT-PCR was performed by using A.B.T.™ 2X qPCR SYBR-Green MasterMix (Atlas Biyoteknoloji, Türkiye) in an Applied Biosystems™ StepOnePlus™ Real-Time PCR System (Thermo, USA). The samples underwent two-step amplification with an initial step at 95°C (10 min), followed by 95°C (15 s), 72°C (30 s), and 60°C (30 s) for 40 cycles. The melting curve was analyzed. Each sample was performed in triplicate independently. GAPDH was used as an internal control (Table 1).

**Table 1.** Primer sequences of the genes used in this study

| Gene Names | Primer Sequences | |
|---|---|---|
| | Forward | Reverse |
| **GAPDH** | GTCTCCTCTGACTTCAACAGCG | ACCACCCTGTTGCTGTAGCCAA |
| **SIGLEC-3** | GTGACTACGGAGAGAACCATCC | GCTGTAACACCAGCTCCTCCAA |
| **SIGLEC-5** | CTCACCTGTCAGATGAAACGCC | CCGTTCCTGAAGATGGTGATGG |
| **SIGLEC-6** | TTTCACCTGCCGTGCTCAGCAT | ACCAGGGTTGTGATGCTAGCTC |
| **SIGLEC-7** | CTGGTCTTCCTCTCCTTCTGTG | GCATCCTTCATGCCTATGTCTCC |
| **SIGLEC-8** | TGACTGTCTTCCAAGGAGATGCC | CTGTTGACAGCACAGACCAGGC |
| **SIGLEC-9** | CCACGAACAAGACCGTCCATCT | TCTGGGAGTGACAGAGATGAGC |
| **SIGLEC-10** | AACGGAGCGTTTCTGGGAATCG | TCTGAGTCCGTCTCTTCGGTAG |
| **SIGLEC-11** | AGAGTGGCTCTGTCTTCCAGCT | CTGAAGACGACAAGGCAGGAAC |
| **SIGLEC-16** | CAACCAGAGTCGAGAGGTGGAA | CACCCGAAAGAAGTACCATGCC |

### Immunocytochemistry

HVT cells were thawed by adding DMEM/F-12 medium containing 10% FBS and 1% penicillin/streptomycin mixture. Thawed cells were monitored every day. Cells reaching 80-90% confluency were passaged the following days. The replicated cells were seeded on chamber slides with 40.000 cells in each well. After the inoculated cells adhered, the medium was removed and fixed with methanol (-20°C). $H_2O_2$ was added to the dried cells, protecting them from light. After 30 minutes, they were washed with Phosphate Buffer Saline (PBS) (CAPRICORN). Secondary kit A solution was applied for 10 minutes. *Siglec 6* and *Siglec 9* (1:100) primary antibodies were applied and incubated at 4°C for one night. After washing with PBS, secondary kit B solution was applied for 1 hour. The chamber slide was rewashed with PBS and exposed to secondary kit C solution for 30 minutes. Stained with 3.3 diaminobenzidine (DAB) and hematoxylin as counterstaining, the chamber slide was covered with entellan. Immunoreactivity against *Siglec-6* and *Siglec-9* was evaluated separately according to the intensity of brown color. HVT cells were scanned at 40X magnification. *Siglec-6* and *Siglec-9* expression was evaluated in the cell nucleus and cytoplasm. Samples were evaluated by light microscopy according to the extent of coloured cells (0%=score 0, 1-10%=score 1, 11-50%=score 2; >51%=score 3) [29, 30].

### Statistical analysis

Data are given as Mean ± Standard Deviation (for each data). The difference between the groups were analyzed using a one-way ANOVA test with GraphPad Prism 9 software.

## Results

### qRT-PCR findings

We observed the melting curves by qRT-PCR amplification targeting Siglec genes using RNA extracts from the HVT cell line. The amplicons specificity was verified by analysis (60 to 95°C) after 40 cycles, except for *Siglec-8* (Figure 1).

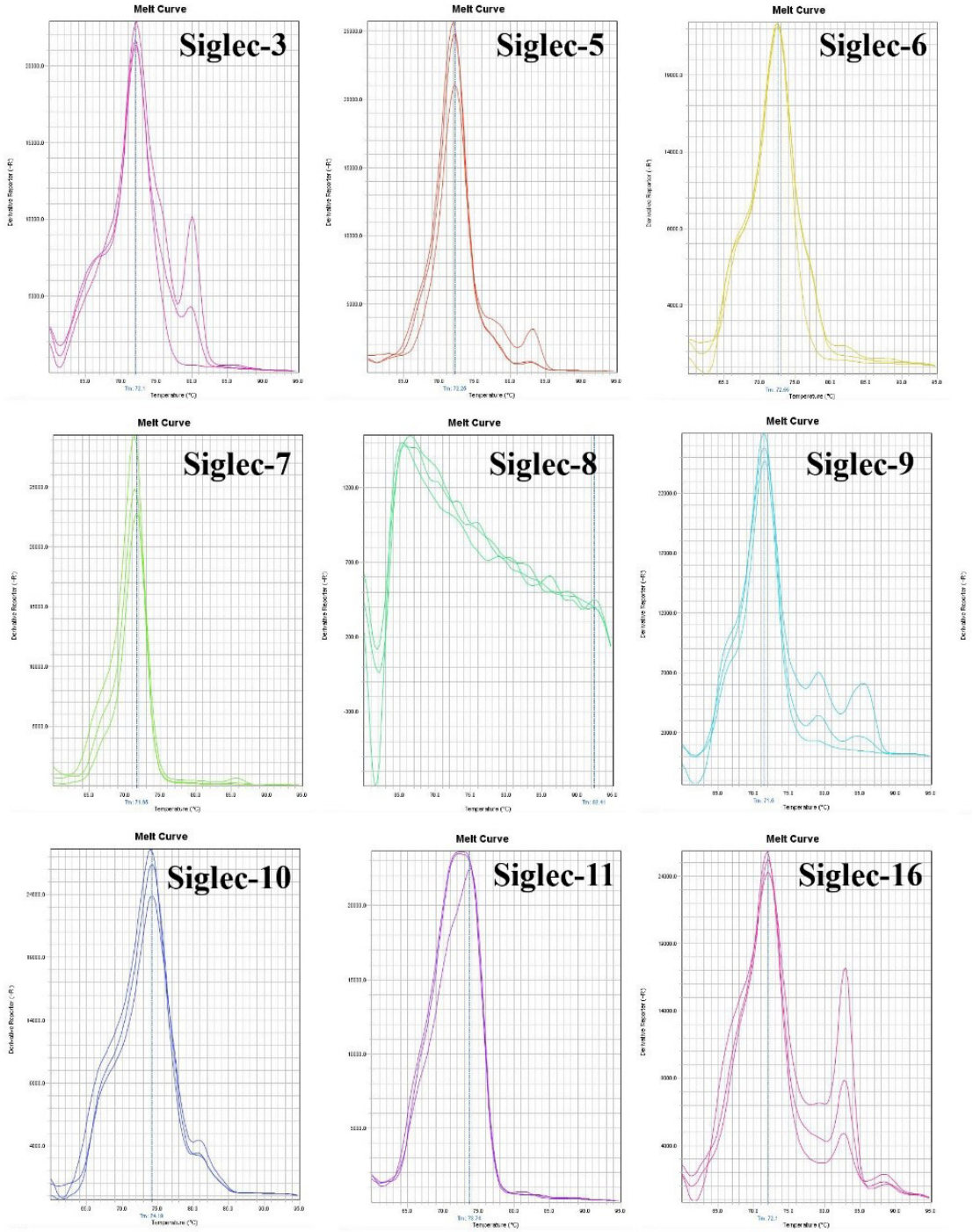


**Figure 1.** Melting curve profile of Siglec genes

The cycle threshold (Ct) values were obtained from each reaction with primer pairs. *Siglec -3, -5, -6, -7, -9, -10, -11*, and *-16* genes were found to be expressed in the HVT cell line. Differently, *Siglec-8* results were undetected after cycle 40, which was considered a negative result (Figure 2).

### Immunocytochemical findings

In the immunocytochemical examination of the HVT cell line revealed that *Siglec-6* expression was moderate in the cytoplasm (Score:2), nuclear staining was weak, and there was no staining in some areas. Along with moderate staining in the cytoplasm and intense staining in some areas, moderate staining in the nuclear membrane in some areas was striking (Figure 3). In The immunocytochemical examination of the HVT cell line, *Siglec-9* expression was prominent in the cytoplasm (Score:3). Nucleus staining was weak, and some areas showed no staining. Nuclear membrane staining was moderate (Score:2) (Figure 3).

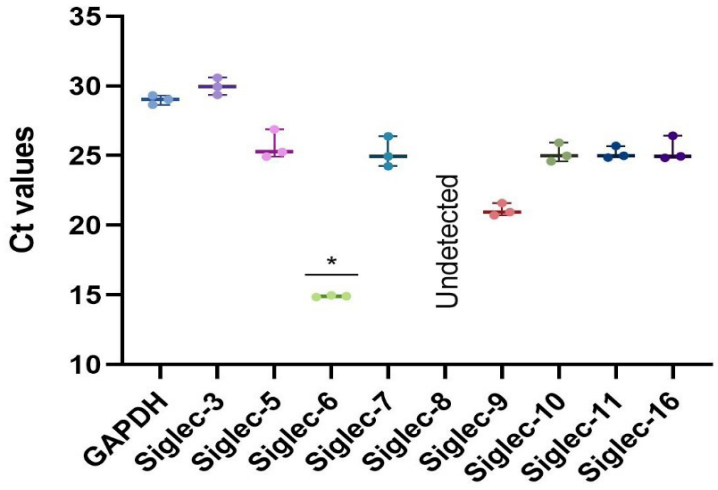


**Figure 2.** qRT-PCR Ct values for reference genes. Expression data is displayed as Ct values for each reference gene in HVT cells. Error bars represent the mean ± standard error of three replicates * indicates statistical difference ($p$<0.05, one-way ANOVA)

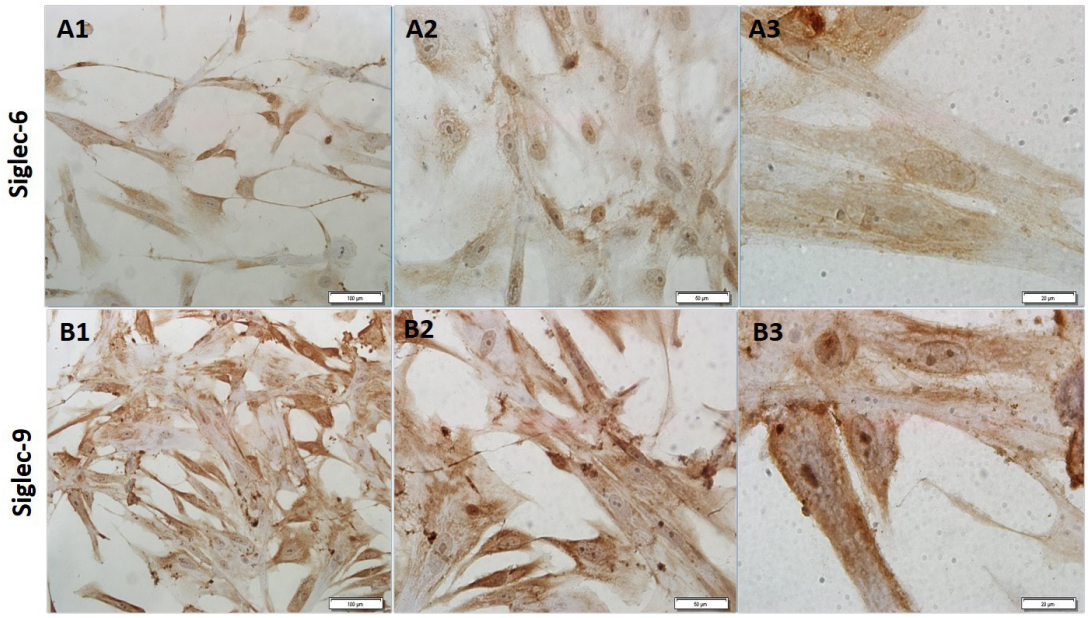


**Figure 3.** Immunocytochemical demonstration of Siglec-6 and Siglec-9 expression in HVT cells A1, B1: 20X; B2, B2: 40X; A3, B3: 100X, Immunoperoxidase & Hematoxylin

## Discussion

The sialic acid-Siglec axis has been partially explored in reproductive system function and embryo implantation. Sialic acids are found in circulating glycoprotein hormones (FSH, LH, hCG). The N-glycans of FSH and hCG are coated with sialic acid, whereas in LH, a different modification occurs with the addition of sulfate. The fate of circulating LH is determined by these glycosylation changes; a specialized receptor in the liver clears the sulfated molecules. This clearance determines the half-life of the hormones in circulation. As a result, the reproductive cycle is optimized [27]. Sialic acids affect fertilization during sperm-egg contact [31]. It has been shown that these glycans contribute to fertilization during interactions with the surfaces and various fluids of the female reproductive system before the sperm arrives the ovum [32]. The endometrium has an agglutinin that detects sialic acids [33]. Sialic acids appear to be affect fertilization [34]. Sialic acids also affect embryogenesis. Although cells in culture can survive and divide without sialic acids, early embryonic death occurred in mice that were genetically engineered to eliminate sialic acid production [35]. Tecle et al. [36] investigated the expression of various Siglecs in pre-menopausal human uterine tissues. They showed that the epithelium primarily expressed *Siglec-10*. They investigated Ishikawa and HEC-1B, for the expression of Siglec on the cell surface. Their analysis revealed that HEC-1B and Ishikawa express *Siglec-10* and *Siglec-11* and activate other Siglecs. Importantly, these two cell lines were found to express *Siglec-16* highly [36]. In a study, despite *Siglec-11* and *Siglec-16* being expressed by endometrial cell lines, *Siglec-11*-Fc and *Siglec-16*-Fc proteins did not bind to human semen [37]. In our study, the presence of *Siglec-10, -11*, and *-16* in HVT was demonstrated in gene analysis using the Real-Time PCR method.

*Siglec-8* is expressed on human mast cells and eosinophils and has low expression on basophils. These cells contribute to allergic and non-allergic diseases. They contribute to the inflammatory response by releasing mediators that attract other cells and activate inflammation. Early studies found that *Siglec-8* binds to monoclonal antibodies or selective sialoglycan ligands, ultimately inducing cell death by eosinophils and inhibiting degranulation of the mast cells. In vivo administration of anti-*Siglec-8* antibodies to the in vitro results were confirmed by transgenic mice expressing *Siglec-8* on eosinophils and mast cells [38]. We did not demonstrate Siglec-8 expression in trophoblast cells in our study.

*Siglec-6* is expressed in syncytiotrophoblasts, cytotrophoblasts and extravillous trophoblasts in the human placenta. It is expressed in B cells of primates. However, placental expression of human is specific. Overexpression of *Siglec-6* was observed in placentas with preeclampsia (PE) patients [39]. Another study showed that expression of *Siglec-6* was higher in PE placentas compared to controls at preterm period. *Siglec-6* expression was approximately twice as high in PE samples in the basal plate and chorionic villi. With this study, *Siglec-6* was thought to have a role in trophoblast differentiation defects. Therefore, it was concluded that overexpression of *Siglec-6* in PE placentas may be a marker of PE [40]. Another study was reported that while *Siglec-6* is expressed in the immune cells of both humans and great apes, its expression in the placenta is human-specific. In the monkey placenta, its expression is either very low or absent. Natural ligands for *Siglec-6* are expressed in the human placenta. Ligands have also been located in the uterine endometrium and trophoblastic or endometrial-origin cell lines. As a result of the immunohistochemical analysis for *Siglec-6* localization in human placentas, a wide expression range was recorded in placentas obtained with normal delivery, with the highest expression [41]. Our study supports these studies, as shown both by Real-Time PCR results and immunocytochemically. In the images obtained immunocytochemically, a prominent reaction was observed in the cytoplasm and nuclear membrane.

Myeloid cells, B cells, NK cells, T cells express *Siglec-9*. It is a cell surface transmembrane receptor. When *Siglec-9* binds to its sialic acid-containing ligands, it initiates a negative signaling cascade that inhibits the function of immune cells. *Siglec-9*-mediated inhibition of the immune response is independent of MHC. Therefore, cancer cells can use this mechanism to evade the host immune response. These results indicate that *Siglec-9* is an important glycoimmune negative checkpoint against cancer and virally infected cells [42]. In a meta-

analysis, it was reported that expression of *Siglec-9* is altered in cancers and is associated with patient survival. The study revealed a correlation between *Siglec-9* expression and clinical characteristics of tumor patients. *Siglec-9* is strongly associated with the tumor immune microenvironment. *Siglec-9* expression levels have been shown to be highest in blood, spleen, and lung tissues and lowest in muscle and bone marrow [43]. Another study showed that *Siglec-9* was expressed in mesothelial cells and connected with glycan epitopes in endometrial cells. Inhibition of *Siglec-9* function decreases the connection between the endometrium and mesothelium [44]. According to Real-Time PCR results, *Siglec 9* expression was found to be considerably higher than other Siglecs, such as *Siglec 6* in our study. Immunologically, it was supported significantly by cytoplasm and nuclear membrane staining.

In conclusion, *Siglec -3, -5, -6, -7, -9, -10, -11*, and *-16* genes were found to be expressed in the HVT cell line in the study. The results showed that *Siglec-6* and *Siglec-9* were expressed more than other Siglec proteins in the HVT cell line. Immunocytochemistry results also support these findings. Our study is the first to show *Siglec-9* expression in human trophoblast cells. Experimental and human placental studies are needed to elucidate the effects of Siglec genes, which are thought to promote cell-cell interactions and regulate the immune system through glycan recognition, on these mechanisms.

**Conflict of interest:** No conflict of interest was declared by the authors.

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**Ethics committee disclosure:** In the our study “Expression of Sialic Acid Binding Receptors (Siglecs) in Human Trophoblast Cell Line”, human first trimester trophoblast cells (Human Trophoblast Cell Line) that we have in stock were used. Cells are cultured in medium. Cells that become confluent between 2 and 3 days are multiplied by changing the medium, and experimental groups of cells that have reached sufficient density are formed. Ethics committee approval is not required for cell culture studies and cells are in our stock.

**Contributions of the authors to the article**

I.V.F. constructed the main idea and hypothesis of the study. I.V.F., N.C. and D.M. developed the theory and arranged/edited the material and method section. D.M. evaluated the data of qRT- PCR in the results section. G.A.M. and N.C. contributed to the histological and immunohistochemical evaluation of the results. The article written by I.V.F., N.C., U.C., C.K. and D.M.

I.V.F. and G.A.M. reviewed the article and made the necessary corrections and approved it. In addition, all authors discussed the entire study and approved the final version.