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Transcriptional Regulation of the Natural Cytotoxicity Receptor NKp44 Gene in Human NK Cell Leukemia

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Abstract

The natural cytotoxicity receptor NKp44 is an important regulator of NK cell function. We have previously reported that NKp44 has binding affinity to heparin and the transferrin containing multimeric sialyl Lewis X produced by human hepatoma HepG2 cells (HepTF) is more highly expressed than the other natural cytotoxicity receptors. To further understand the differences between natural cytotoxicity receptors, we sought to understand the gene transcriptional regulation of NKp44 in KHYG-1 human NK leukemia cells. 5′-rapid amplification of cDNA ends revealed the location of the NKp44 transcription initiation sites allowing identification of the 5′ untranscribed region. The result of the dual luciferase assay suggested that regulation of NKp44 gene expression is affected by the -1963 to -1599 nt region and the -352 to -231 nt region relative to the transcription initiation sites. The -410 to +1 nt region contained potential binding sites for the transcription factors AP-1, Oct-1, HNF-4, and Pax-4. These results suggest that defined regions in the 5′-flanking region of NKp44 gene is important for NKp44 transcription and regulation.

Keywords: Natural cytotoxicity receptor; NCR2; NKp44; Promoter; Transcriptional regulation

Introduction

Natural Killer (NK) cells are important for natural immunity against tumors and virally infected cells. The Natural cytotoxicity Receptors (NCRs) NKp46 (NCR1, CD335), NKp44 (NCR2,CD336), and NKp30 (NCR3,CD337) have immunoglobulin-like structure. These receptors are classified as the Immunoglobulin (Ig) superfamily. NCRs are characterized by the presence of large extracellular Ig-like domains, transmembrane regions containing positively charged amino acids, and short cytoplasmic tails that lack Immunoreceptor Tyrosine-based Activating Motifs (ITAM) [1-3]. NCRs can, however, associate with adaptor molecules that contain an ITAM. NCRs produce an activation signal via an adapter molecule. In this regard, NCRs associate with a negatively charged aspartate residue in the transmembrane regions of different ITAM-containing signaling proteins through their positively charged transmembrane residues. NCRs trigger the cytotoxicity of NK cells towards target cells lacking expression or with less expression of the major histocompatibility complex (MHC) class I molecule.

NKp44 expression is induced by stimulation such as that by interleukin (IL)-2 [4], whereas resting and activated NK cells express NKp46 [5,6] and NKp30 [7]. Signal transduction from NCRs is amplified because of selective cross-talk among different NCRs [8].

Immunoglobulin-conjugated NKp46D2 and NKp44 bind to ligands such as influenza virus hemagglutinin and Sendai virus hemagglutinin-neuraminidase via the 2,6-NeuAc residue on NKp44; however, this NeuAc residue is not necessary for NKp44 interaction with cancer cells [9-11]. NKp44 can also bind to *Mycobacterium bovis* bacillus Calmette Guerin [12] and envelop glycoproteins from West Nile and Dengue viruses [13]. Microarray and surface plasmon resonance analysis using heparin oligosaccharides have shown that NKp44 binds to negatively charged and IdoA2SGlcNS6S-containing heparin oligosaccharides with a different binding pattern compared to NKp46 and NKp30, which have similar binding patterns to each other [14,15].

We previously reported that the cytotoxic activity of IL-2-activated KHYG-1 cells is elevated by fucosyltransferase (FUT)-3 gene-transfected K562 (K562/FUT) cells, which express the sialyl Lewis X (sLeX) antigen (NeuAc2, 3Gal1, 4(Fuc1,3)GlcNAc-R) at high levels [16]. Furthermore, we have reported that extracellular domains of NKp46 and NKp44 tagged with hexa-histidine, synthesized as a recombinant protein, can bind to transferrin containing multimeric sialyl Lewis X produced by human hepatoma HepG2 cells (HepTF), and that positively charged amino acids in NKp46 and NKp44 are essential for binding glycan ligands [17,18]. Previously, we compared the binding affinity of natural cytotoxicity receptors to their ligands by determining Kd values, demonstrating that NKp44 binding affinity to heparin and multimeric sLeX on HepTF is higher than that of the other natural cytotoxicity receptors [17,18].

Currently, there are very few detailed reports describing the transcriptional regulation of NKp44, which is surprising, given that it is well known to be inducible. In this report, we have studied the transcriptional regulation of NKp44 in a human NK leukemia cell line (KHYG-1) using 5′-rapid amplification of cDNA ends, a dual luciferase assay to analyze sequential deletions of the 5′ untranscribed regions.

Materials and Methods

Cells and cell culture

The human NK leukemia cell line (KHYG-1) was purchased from the Japanese Collection of Research Bioresources Cell Bank (Tokyo,

Japan) and cultured in Roswell Park Memorial Institute (RPMI) medium 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (JRC Biosciences, Lenexa, KS, USA) in the presence of 100 U/mL rIL-2 (Shionogi Co., Osaka, Japan) under a humidified atmosphere containing 5% CO_2 at 37°C.

Determination of the NKp44 transcription initiation sites in KHYG-1 using 5′-rapid amplification of cDNA ends (5′-RACE)

The 5′ end of the NKp44 cDNA was amplified using a 5′-Full RACE Core Set (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg of total RNA using the 5′-phosphorylated NKp44-specific primer 5′-TGTGTGGAGGCAGA -3′. Template mRNA was digested with RNase H at 37°C for 30 min and the cDNA was precipitated by the addition of ethanol. The single-strand DNA precipitate was dissolved in ligation buffer and incubated with T4 ligase at 16°C for 16 h. The concatemer DNA was used as a template for the first PCR amplification, using a forward primer 5′- GCTTGGACCTCTCGATTCAC-3′ and a reverse primer 5′- GCCTCCTTACACCAGCCTTT-3′. PCR conditions were 94°C for 3 min followed by 25 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s. The resulting PCR products were diluted 100-fold with distilled water, and amplified under the conditions described above, using a second forward primer 5′-TGATGCTGGCTTCTTCACTG-3′ and a second reverse primer 5′-ATCTCACGGTTAGCGTCTGC-3′. PCR products were ligated into the pGL4 vector (Life Technologies) and sequenced using a 3730xl DNA analyzer (Applied Biosystems, Waltham, Massachusetts, USA).

Deletion constructs of plasmids and luciferase assay

The 5′-flanking region (-1963 to -1 nt) of the transcription initiation site of the NKp44 gene was amplified from KHYG-1-derived genomic DNA by PCR using the forward primers 5′-CAGGAGTCCAGGGGCAGGCA-3′ (-1963nt) and 5′-GAGGAAGCTGAGCGTGCAGATGG-3′ (-973 nt), and the reverse primer 5′- GGCCTGGGTTGGGGAGATGG-3′. The two PCR products were 5′-phosphorylated using T4 polynucleotide kinase (Toyobo, Osaka, Japan) and ligated into the pGL4 vector, which was digested with EcoRV (Toyobo) and treated with E. coli alkaline phosphatase (Toyobo). The sequence of the cloned NKp44 transcription initiation sites was confirmed using a 3730xl DNA analyzer.

Plasmids encoding deletions of the NKp44 transcription initiation sites were constructed by PCR to contain the following transcription initiation site sequences; pGL4.11/-1963 to -1, pGL4.11/-1598 to -1, pGL4.11/-1263 to -1, pGL4.11/-973 to -1, pGL4.11/-352 to -1, pGL4.11/-230 to -1, and pGL4.11/-153 to-1. To achieve this, the GL4/-1963 to-1 plasmid was used as the PCR template, and the individual PCR fragments were ligated into the parental pGL4.11 vector. PCR conditions were 94°C for 2 min, followed by 30 cycles of 95°C for 0.5 min, 60°C for 5 min, and 72°C for 1 min. The specific forward primers used were as follows:

5′- GAGGAAGCTGAGCGTGCAGATGG-3′ (pGL4.11/-1963 to -1),

5′-ACACACACACACGGTGACG -3′ (pGL4.11/-1598 to -1), 5′-GTAATGTCTCCCTCGCGAAC-3′ (pGL4.11/-1263 to -1),

5′-CAGGAGTCCAGGGGCAGGCA-3′ (pGL4.11/-973 to -1),

5′- AGTGCCCCTGAGTTTGTCAT-3′ (pGL4.11/-352 to -1), 5′-CGCCCACAGTTTCCTCCTGGCCTTT-3′ (pGL4.11/-230 to -1), and

5′-GGGAGAGCAGGCATCTTCTA-3′ (pGL4.11/-153 to -1). The specific reverse primer used was 5′- ATCCTCGAGGCTAGCG-3′.

For the NKp44 promoter assay, 9 μg of the different pGL4.11 constructs and 0.02 μg of the pRL-TK-Luc vector (Promega Co., Madison, WI, USA) were transiently co-transfected into KHYG-1 cells (15×10^5 cells per well) using Xfect transfection reagent (TaKaRa Bio Inc, Shiga, Japan). The pRL-TK-Luc vector contains the TK promoter 5′-upstream of the *Renilla luciferase* gene. After 48 h, the cells were harvested and lysed. Firefly and *Renilla luciferase* activities were determined using the Dual Luciferase Assay System and measured using a 20/20n luminometer (Promega).

Results and Discussion

Determination of the NKp44 transcription initiation site in KHYG-1

To understand the constitutive expression of the NKp44 gene in KHYG-1 cells, we determined its transcription initiation sites using 5′-RACE, which revealed one major transcript of about 450 bp as shown in Figure 1. To determine the sequence, obtained PCR products were ligated into the pGL4 vector and sequenced. The transcription initiation sites and the 5′-untranscribed region of NKp44 genomic DNA are shown in Figure 2.

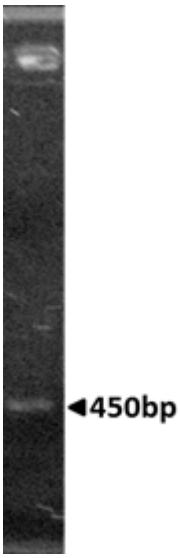

Figure 1: Determination of the NKp44 transcription initiation sites in KHYG-1 using 5′-rapid amplification of cDNA ends (5′-RACE), Using extracted total RNA from KHYG-1 cells, the NKp44 gene transcription initiation sites was determined using 5′-RACE, revealing one major transcript of about 450 bp.

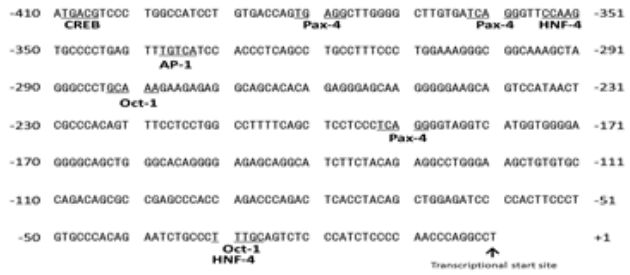

Figure 2: Nucleotide sequence of the transcription initiation sites and the 5′-untranscribed region of NKp44 genomic DNA, PCR products obtained in the 5′-RACE analysis were ligated into the pGL4.11 vector and sequenced. Nucleotides are numbered with the transcription initiation sites determined in the 5′-RACE analysis as +1. Transcription factor binding motifs were present in the 5′-flanking region of the NKp44 gene and are shown underlined for AP-1, Oct-1, HNF-4 and Pax-4.

Promoter activities of deletion constructs of the NKp44 5′-flanking region

To understand promotor region of the NKp44 gene in KHYG-1 cells, we prepared NKp44 5'-flanking region deletion constructs using pGL4.11/-1963 to-1 as the PCR template. And the constructs ligated into pGL4.11. After co-transfection of the deletion constructs with the pRL-TK-Luc vector into KHYG-1 cells, dual luciferase activities were determined (Figure 3). Firefly luciferase activity markedly decreased on deletion of the region from -1963 to -1599 nt and on deletion of the region from -352 to -231. Using the Match program (http://gene-regulation.com/), homology search revealed that the 5′-untranscribed region (-1963 to -1599) of the NKp44 gene contained several binding sites for transcription factors such as v-Maf, MyoD, TCF11, and AP-1. In contrast, the -410 to -1 untranscribed region contained binding sites for AP-1, Oct-1, HNF-4 and Pax-4. The -410 to -1 untranscribed region of the NKp44 gene likely represents the NKp44 gene promoter because the promoter is typically found in the neighborhood of the transcription initiation sites. In comparison, the -1963 to -1599 5′-untranscribed region of the NKp44 gene may contain putative enhancer binding sites. These data suggested that transcription of the NKp44 gene in KHYG-1 was constitutively controlled by the -410 to -1 region, which contained binding sites for the transcription factors AP-1, Oct-1, HNF-4 and Pax-4.

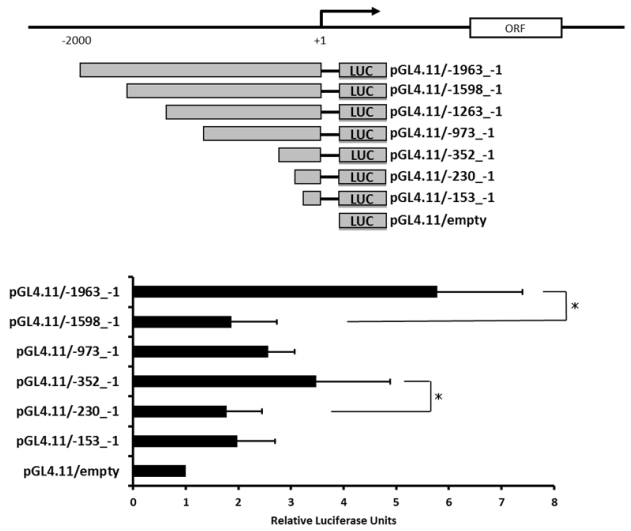

Figure 3: Promoter activities of the NKp44 reporter gene deletion constructs in KHYG-1 cells, Luciferase reporter plasmids containing the 5′ untranscribed region of the NKp44 gene and deletions thereof were prepared by amplification of KHYG-1 genomic DNA and ligation into pGL4.11. The promoter deletion reporter plasmids and pRL-TK-Luc were then co-transfected into KHYG-1 cells, and following 48 h of incubation, the dual luciferase activity was measured. To correct for differences in transfection efficiency, the results are normalized to Renilla luciferase activity. The relative activities of luciferase are normalized to the activity of pGL4.11 itself set as 1. The results obtained in three independent experiments are expressed as mean ± SD. Significant differences ($P<0.05$) are indicated by asterisks.

NK cells are important for natural immunity against tumor and virally infected cells. These functions are regulated through various receptors on NK cells [19-22]. NKp44 is an important receptor in NK cells that has been shown to bind to Glycosaminoglycans (GAGs) such as heparin and sulfated polysaccharides, and has also been shown to bind the multimeric sialyl Lewis X residue [18]. These ligands are known to have altered expression levels in some diseases such as cancer, and a range of viral diseases [23-26]. In recognizing these ligands, NKp44 is important for the regulation of NK cell function.

In this report, we have identified the transcription initiation sites of the NKp44 gene using 5"-RACE. Deletion of nucleotides in the regions -1963 to -1598 nt and -352 to -231 nt upstream of the transcription initiation sites reduced transcription of the NKp44 gene in KHYG-This result suggests that a putative enhancer is contained within the -1963 to -1599 nt region and that the promoter is contained within the -352 to -231 nt region. Furthermore, both of these regions appear to be involved in the regulation of NKp44 gene expression in KHYG-1 cells. The 5′-untranscribed region (-410 to -1) of the NKp44 gene contained sequence with ability to bind for the transcription factors AP-1, Oct-1, HNF-4 and Pax-4.

To date, the regulation of expression of only a few NK receptor genes in NK cells has been reported including for NKp46 and CD94, a known C-type lectin-like receptor. The NKp46 gene has two regulatory elements in its 5′-flanking region, one of which contains a binding site

for the runt-related transcription factor (RUNX). In fact, the NKp46 gene reported to be regulated by RUNX3 [27]. The human CD94 gene contains potential binding sites for STAT and Ets, suggesting that these transcription factors regulate CD94 gene expression [28].

Regulation of receptor expression on NK cell is important in understanding their role in immunity. Further studies are therefore required to understand the transcription factors essential for NKp44 transcription and ultimately the factors and pathways that control the cell surface expression of NKp44.

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Conflict of Interest

The authors declare no conflict of interest. The authors alone are responsible for the content of the paper.

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