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Characterization of 5′ promoter and exon 1–3 polymorphism of the RAET1E gene

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ABSTRACT

NKG2D is an activating receptor utilized by natural killer (NK) cells that recognizes upregulated ligands on infected, tumorigenic and damaged cells, leading to their cytolysis. However, the NKG2D ligand (NKG2DL) system is very complex with eight known gene loci encoding slightly different molecules. Furthermore, most NKG2DL gene loci such as MICA and MICB are highly polymorphic with potential for functional differences. NKG2DL expression on tumors varies depending on the malignancy and tumors can also release soluble NKG2DL that exert anergic effects on NK cells when engagement with NKG2D occurs, allowing escape from NK cell immunosurveillance.

We carried out RAET1E typing of IHW cell line DNA, including a 580 bp proximal promoter fragment and exons 1–3 identifying 13 of 15 known RAET1E alleles. We determined 7 polymorphisms within the promoter region, including 2 already known that contributed to 9 promoter types. RAET1E alleles with variability in the extracellular region also differed with respect to promoter type and one allele, RAET1E*003, associated with 5 promoter types. We then identified putative transcription factor binding sites for RAET1E, and found 5 of the 7 promoter polymorphisms may disrupt these sites, abrogating binding of transcription factors and varying the potential level of expression.

1. Introduction

Among the activating receptors expressed by natural killer (NK) cells, the NK group 2, member D (NKG2D) receptor has been extensively studied owing to its potency and importance in viral and tumor recognition [1]. NKG2D ligand (NKG2DL) expression is limited but upregulated by tumorigenesis, injury or viral infection, heralding cytolysis of the 'stressed' cell [2]. However, soluble NKG2DL interacting with NKG2D initiates blocking and downregulation of this receptor, rendering NK cells hyporesponsive [3,4]. Thus, NKG2DL may induce both activation and inhibition signals to regulate the activity of NK cells. However, this mechanism is confounded by the existence of multiple types of NKG2DL encoded by eight genes [5–7] and expression also differs according to the type of cell or tissue [8]. Furthermore NKG2DL gene loci are polymorphic and alleles differ between individuals and ethnic groups with the possibility of variable NKG2DL recognition and potential level of activation or inhibition [9–12].

NKG2DL can be categorized into two broad groups. The MHC class I-related chain A and B (MICA/B) were the fist characterized NKG2DL [2,13] and have received the most attention owing to their highly polymorphic nature, possible relevance in transplantation allorecognition, cancer and association with autoimmunity [2]. The second group includes the unique long 16 binding proteins (ULBP1–6), also known as retinoic acid early transcript 1 (RAET1) and these loci are also polymorphic, although less diverse than MICA/B [14]. Variable expression of NKG2DL is reported in association with various malignancies [8] such as MICA/B expression on pancreatic, prostate and breast cancer tumors [15], ULBP1 on leukemia and lymphoma cells [16], ULBP2 on melanoma cells [17] and ULBP3 on mesothelioma cells [18]. In most cases high levels of expression correlate with good prognosis as NK cells are able to efficiently eliminate these cells. However, tumors and also viruses can circumvent NK cell cytotoxicity by hijacking cellular mechanisms to generate soluble NKG2DL (sNKG2DL) by protease

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cleavage [19], alternative splicing [20] or exosome release [21]. Protease cleavage of cell surface NKG2DL not only produces sNKG2DL that can render NK cells hyporesponsive but reduces the number of NKG2D targets on the affected cell. We have also found sNKG2DL in cord blood plasma that are capable of NK cell inhibition, possibly representing a fetal-maternal tolerance mechanism [22]. Other mechanisms allowing viral escape from immunosurveillance include prevention of cell surface NKG2DL expression by retention within the cell, a mechanism successfully exploited by human cytomegalovirus (hCMV) [23].

Polymorphism of NKG2DL probably arose through evolution as variants are selected that are more resistant to manipulation. For example hCMV prevents expression of MICA alleles, except the prevalent MICA*008. This is due to a single nucleotide deletion resulting in a premature stop codon and a truncated TM domain, preventing sequestration by hCMV viral protein UL142 [23]. It has recently been shown that hCMV has coevolved to target the escape variant MICA*008 using viral glycoprotein US9 to prevent its expression [24], highlighting the dynamic nature of 'cat and mouse' evolution. It is probable that some of the extensive variation in NKG2DL sequence and structure enables fine-tuning of the immune response in different tissues. The level of expression is also important as very high or very low NKG2DL expression is ignored by NK cells and so too is persistent or chronic expression [25]. Therefore, an efficient NK cell activation requires sudden, optimal NKG2DL upregulation, governed by transcriptional control.

RAET1E (ULBP4) is a functional ligand for NKG2D that does not bind UL16 [26], therefore the official HGNC name RAET1E is used throughout. Thought to be human orthologs of the murine *Raet1a-d* genes, RAET1 gene loci probably derived by serial gene duplication. RAET1E has the most divergent sequence with <43% homology [5] to other RAET1 loci but remains among the least studied and characterized NKG2DL. A notable feature shared with RAET1G is the presence of a TM region and a hydrophilic cytoplasmic tail [5], similar to MICA/B in humans and H60 or MULT-1 ligands in mice [27]. In contrast to other RAET1 loci, RAET1E expression is limited to the skin [26], but can also be expressed on ovarian tumors [28]. Interestingly RAET1E (and ULBP2) expression by ovarian cancer inversely correlates with disease survival [28], which is not the case for other malignancies. Soluble RAET1E molecules are generated by alternative splicing, a mechanism shared with RAET1G to produce RAET1E2 [20] and RAET1G2 [29] respectively that, like other soluble NKG2DL, downregulate NKG2D expression and reduce NK cytotoxicity [20]. Based on sequences of exons 2–3, there are 11 known alleles (RAET1E*001–011) [10,30]. RAET1E*006 and *007 are unique to northeastern Thais and RAET1E*009 is unique among Kolla American Indians [30].

For this study, we extended SBT strategies to include up to 580 bp of proximal promoter sequences of RAET1E alleles and also included exon 1. Five novel polymorphisms were found in the promoter region and we show that RAET1E alleles can have either one type of promoter sequence or several, a characteristic shared with MICA/B alleles [31]. We also analyzed the promoter sequences for putative transcription factor binding sites (TFBS) potentially regulating RAET1E expression and determined whether any promoter polymorphisms disrupt TFBS that may alter transcription.

2. Materials and methods

2.1. Cell line DNA

RAET1E allele (exons 1–3) and promoter polymorphisms were determined using reference DNA samples. DNA was extracted from IHW B lymphoblastoid cell lines (IHW BLCLs) [32] using an 'in-house' salting-out technique. IHW cell line DNA from our panel was chosen to include mostly European ethnicity with some North American and American Indian samples. We also included details of some cord blood samples that were found to contain rare alleles not seen from IHW cell line DNA typing. All allele and promoter types were verified by sequence-based typing (SBT) of the RAET1E gene as illustrated in Fig. 1. Allele and promoter combinations were determined using 60 IHW cell line, 7 Kolla American Indian and 6 cord blood DNA samples obtained from the Anthony Nolan Cell Therapy Centre, Nottingham, UK.

2.2. RAET1E PCR amplification

Amplification primers were used to amplify a 2344 base pair (bp) fragment of the RAET1E gene including the minimal promoter region and exons 1–3 (Table S1). The 5′ primer RAET1E-5 was situated between −709 to −729 bp and −724 to −744 (depending on sequence length of individual alleles) upstream of the ATG transcription initiation codon of exon 1. The 3′ primer RAET1E-3 was previously described [5] and situated at position 1595 to 1615 of intron 3 (numbering from exon 1). PCR reactions were performed using a PTC-200 thermal cycler (MJ Research, Quebec, Canada) in 25 μl reaction volumes including 125 ng genomic DNA, 12.5 pmol each primer and 1U Takara DNA polymerase (Takara Bio Inc., Japan), according to manufacturer's instructions. Thermal cycling conditions were as follows: 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 67 °C for 1 min 30 s and 72 °C for 2 min 30 s and a final extension step of 72 °C for 10 min. Post-PCR reaction enzymatic purification was performed using Illustra™ Exostar™ (GE Healthcare Life Sciences, Little Chalfont, UK) according to manufacturer's instructions.

2.3. RAET1E promoter and exon 1–3 sequence-based typing

Due to a poly AC sequence in the minimal promoter region that varied in length between alleles, only 580 bp of the promoter sequence were included along with exons 1–3 for heterozygous allele typing. Four primers were used to obtain bidirectional sequencing of the promoter region, including exon 1 and two bidirectional primers were used for each of exons 2 and 3. Details of primers are given in Table S1 and the strategy is illustrated in Fig. 1. Sanger dye terminator sequencing was performed using standard cycle sequencing parameters on an ABI3730xl sequencer (Applied Biosystems, CA, USA). Consensus sequences were analyzed for heterozygous combinations of alleles using MATCHTOOLS 1.0 (Applied Biosystems, CA, USA) with RAET1E promoter sequence libraries. We achieved allele level typing in all cases with no ambiguous allele combinations.

2.4. PCR cloning to isolate individual RAET1E alleles

Following typing, individual RAET1E promoter and allele sequences were obtained from RAET1E homozygous IHW cell lines, where possible. Alleles only found in combination with other alleles were separated after amplification by cloning using pGEM®-T easy vector system II (Promega,WI, USA) before sequencing at least three different clones of the allele of interest, according to manufacturer's instructions.

2.5. Prediction of RAET1E putative transcription factor binding sites (TFBS)

It has been shown that around 500 bp of genomic sequence prior to the transcription start site is sufficient to analyze most known promoter TFBS and regulatory elements [33]. To include a region with variable AC repeats between RAET1E alleles, we