Novel Splice Variants of IL-33: Differential Expression in Normal and Transformed Cells

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TO THE EDITOR

IL-33 is a newly discovered member of the IL-1 family and is constitutively expressed in the epithelial and endothelial cells of various organs (Moussion *et al.*, 2008). IL-33 is important to the T helper 2 (Th2) immune reaction because its receptor, ST2L, is expressed on Th2 lymphocytes, basophils, eosinophils, and mast cells (Schmitz *et al.*, 2005). It may also function independently of the immune reaction (Sanada *et al.*, 2007). IL-33 is usually found in the nucleus, produced as pro-form IL-33 (pro-IL-33), and is digested into a mature form with a lower molecular weight when it is secreted from the cells. Mature IL-33 was first thought to be the active form (Schmitz *et al.*, 2005). However, recent reports have shown that the active pro-form of IL-33 is digested into an inactive mature form (Cayrol and Girard, 2009).

Neonatal foreskin normal human epidermal keratinocytes (NHEKs) were purchased from Kurabo (Osaka, Japan) and cultured in keratinocyte serum-free medium (Invitrogen, Carlsbad, CA) supplemented with bovine pituitary extract (Kyokuto Seiyaku, Tokyo, Japan) and epidermal growth factor (R&D Systems, Minneapolis, MN). HaCaT keratinocytes were a generous gift from Dr Kuroki (Showa University) with the permission of Dr Fusenig (Institute Fur

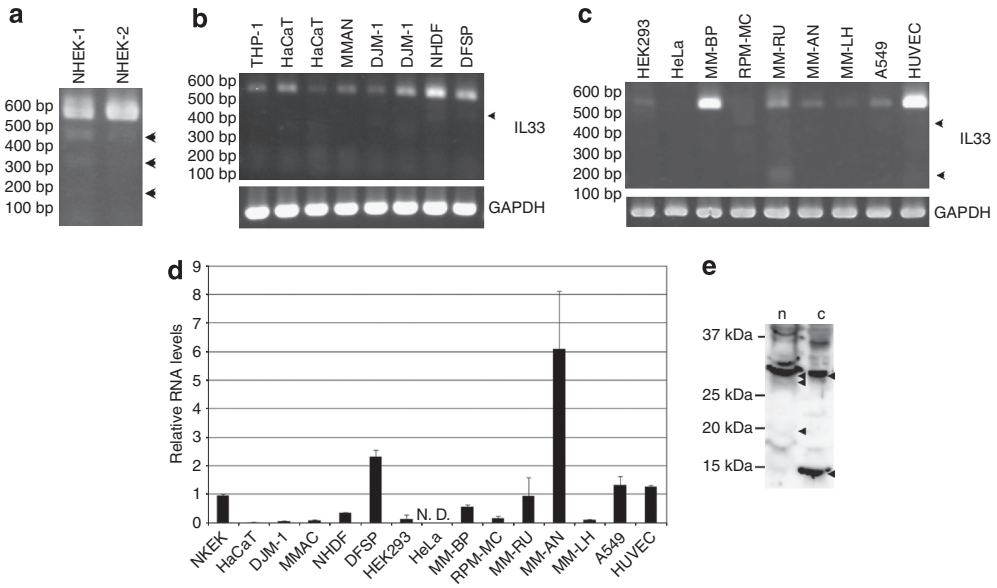

Figure 1. Cells expressing full-length IL-33 messenger RNA (mRNA) at diverse levels. (**a**–**c**) Total RNA (1 μg) was reverse transcribed with a SuperScript III Reverse Transcriptase Kit (Invitrogen). PCR was carried out for 35 cycles of 95 °C (20 s), 56 °C (20 s), and 72 °C (15 s), by TaKaRa Ex Taq polymerase (TaKaRa) using ex1 (5′-AGCCTTGTGTTTCAAGCTGG-3′) and R1 (5′-ATGGAGCTCCACAGAGTGTTC-3′) primers. Products were visualized by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Arrowheads indicate products shorter than full-length IL-33. (**d**) Real-time PCR performed using the TaqMan Real-time PCR System (ABI). The primers and probes for IL-33 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from ABI. Quantities of mRNA were measured relative to GAPDH mRNA levels. ND, not detected. Each bar denotes three independent experiments (mean ± SD). (**e**) Nuclear (n) and cytoplasmic (c) fractions of normal human epidermal keratinocytes were extracted using the Nuclear/Cytosol Fraction Kit (BioVision, Mountain View, CA). Cell extracts were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. IL-33 was detected with anti-human IL-33 antibody (NT; ProSci, Poway, CA).

Abbreviations: DFSP, dermatofibrosarcoma protuberans; FCS, fetal calf serum; HUVEC, human umbilical vein endothelial cell; NHDF, normal human dermal fibroblast; NHEK, normal human epidermal keratinocyte; Pen/St, penicillin/streptomycin; Th, T helper

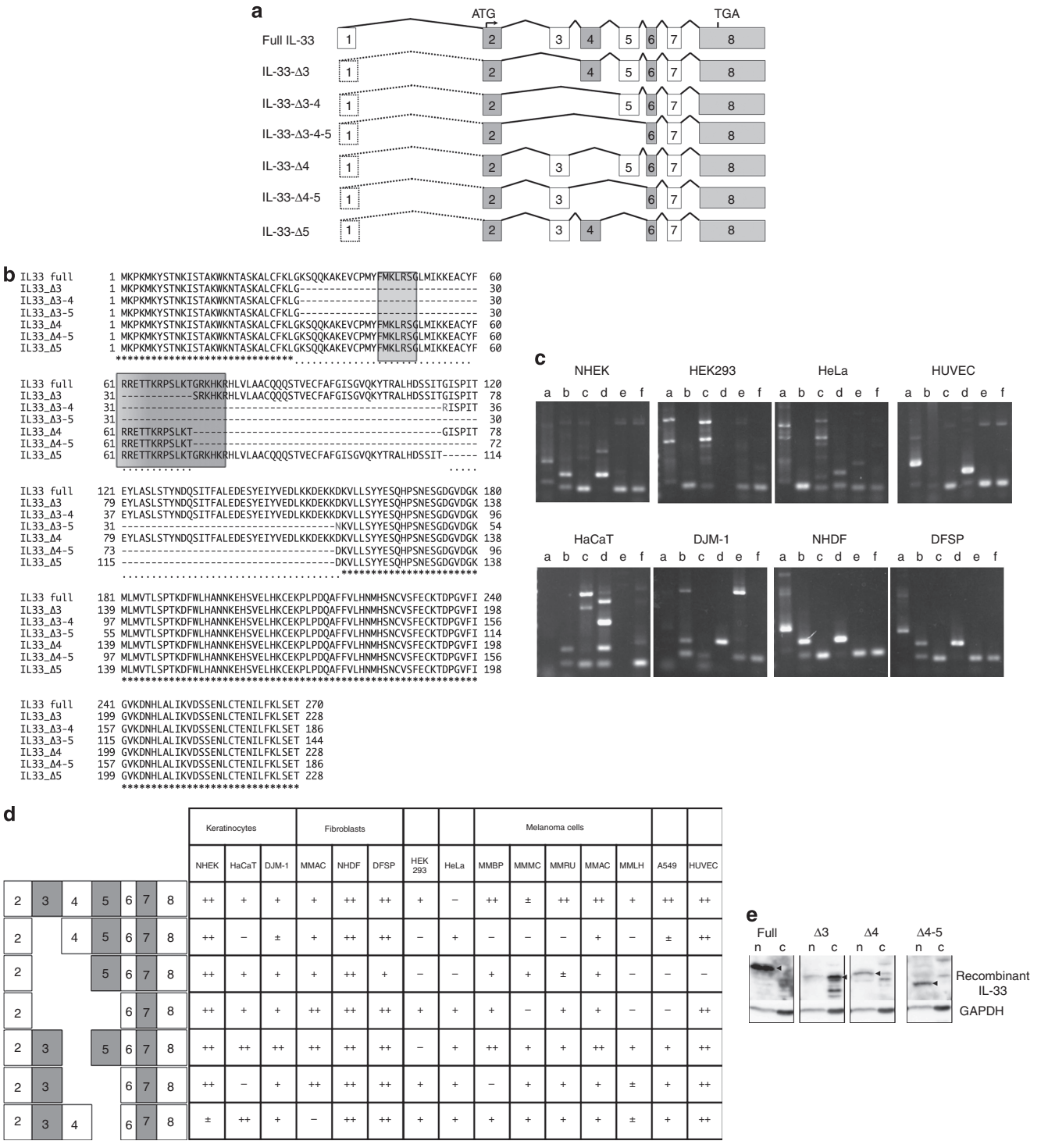

Figure 2. Genome organization, sequence, and expression of IL-33 splice variants in different types of cells. (**a**) Schematic diagram of splicing variant structures. Exon 1 was not validated in this study (dashed box and line). (**b**) Multiple alignment of putative amino-acid sequences. Gray box: binding site to histones H2 and H3; gradient box: putative nuclear-localization signal sequence. (**c**) PCR using primers designed to detect each splicing variant. a: Exon 3 deletion (5′-CAAGCTGGGAAGTAGAAAGCAC-3′), 410 bp; b: deletion of exons 3 and 4 (5′-CAAGCTGGGAAGAATTTCACC-3′), 280 bp; c: deletion of exons 3, 4, and 5 (5′-CAAGCTGGGAAATAAGGTGTTAC-3′), 150 bp; d: exon 4 deletion (5′-CTGAAAACAGGAATTTCACC-3′), 280 bp; e; deletion of exons 4 and 5 (5′-CTGAAAACAGATAAGGTGTTACTG-3′), 150 bp; f: exon 5 deletion (5′-AGTATCACAGATAAGGTGTTACTG-3′), 150 bp. PCR products were loaded into a 1.5% agarose gel and visualized by ethidium bromide staining. (**d**) PCR analysis summary. ++: High expression; +: moderate expression; −: no expression. (**e**) Expression patterns of recombinant IL-33 splice variants. Full-length IL-33 and some IL-33 splice variants were constructed using PCR. Two micrograms of plasmid was transfected into 1×10^6 cells with FuGENE HD Transfection reagent (Promega). Nuclear (n) and cytoplasmic (c) fractions were extracted, and recombinant proteins were detected with anti-FLAG antibody (Sigma, Saint Louis, Missouri). DFSP, dermatofibrosarcoma protuberans; HUVEC, human umbilical vein endothelial cell; NHDF, normal human dermal fibroblasts; NHEK, normal human epidermal keratinocyte.

Zell- und Tumourbiologie, Deutsches Kresforschungszentrum, Heidelberg, Germany). They were grown routinely in Eagle's minimum essential medium (Invitrogen) supplemented with 10% fetal calf serum (FCS) in a humidified CO_2 incubator. We used cells from the 30th to 50th passage. A human squamous cell carcinoma cell line (DJM cells) was established in the Department of Dermatology, Jichi Medical University (Katayama *et al.*, 1991) and cultured in DMEM (Invitrogen) with 10% FCS and 1% penicillin/streptomycin (Pen/St). THP-1 cells were purchased from the ATCC and cultured in RPMI 1640 (Invitrogen) with 10% FCS and 1% Pen/St.

Normal human dermal fibroblasts (NHDF) were purchased from Kurabo. Fibroblasts from dermatofibrosarcoma protuberans (DFSP) were obtained from tumors excised from patients after informed consent was provided. The human melanoma cell lines MM-AN, MM-BP, MM-LH, MM-RU, and RPM-MC were kindly provided by Dr H Randolph Byers (Harvard Medical School). All cell lines were established from metastatic lymph nodes, except for RPM-MC, which originated in a recurrent primary lesion (Byers *et al.*, 1991). HEK293, A549, and HeLa cells were cultured in DMEM with 10% FCS containing 1% Pen/St.

Dr Yanagisawa (Jichi Medical University) kindly provided RNA extracted from human umbilical vein endothelial cells (HUVEC). The primers used in this study were designed on the basis of the full-length IL-33 sequence (GenBank accession no. NM_033439, Baekkevold *et al.*, 2003).

PCR products were extracted from agarose gel using the Wizard SV Gel and PCR Clean-Up System (Promega, Tokyo, Japan). Purified fragments were cloned into the *Eco*RV site of pBluescript SK(−), and sequencing analysis was performed with an ABI 310 Sequencer (Applied Biosystems, ABI, Norwalk, CT) using universal primers.

We attempted to evaluate the messenger RNA expression levels of full-length pro-IL-33 among multiple cell types (Figure 1a–d). NHEKs, DFSP, A549, and HUVEC expressed comparable levels of IL-33, whereas DJM, HaCaT, and HEK293 expressed IL-33 at much lower levels. IL-33 was not detected in HeLa cells. Several malignant melanoma cell lines expressed different levels of IL-33. NHEKs expressed higher levels of IL-33 than its transformed variants did; however, NHDF expressed lower levels of IL-33 than its transformed variant (DFSP cells) did, suggesting that malignant transformation did not drive IL-33 expression levels in a certain direction.

We suspected the existence of smaller splice variants in the IL-33 PCR products, because we observed several smaller bands (Figure 1a–c). Using western blotting, we also detected several IL-33 bands in both nuclear and cytoplasmic fractions (Figure 1e) that were absent in IL-33 knockdown cells (data not shown). We thus purified a single PCR product from each of the smaller bands and determined the respective nucleotide sequences. Sequencing revealed multiple IL-33 splice variants of different sizes, as shown in Figure 2a. These include variants that lack a single exon (3, 4, or 5), as well as variants that simultaneously lack exons 3 and 4; 4 and 5; or 3, 4, and 5.

The amino-acid sequence of each exon did not differ, except for variants in which the deletion began at exon 3 (Figure 2b). The first amino acid following the abnormal splicing event was replaced by a different amino acid in these variants because the splicing event occurred within the first codon of exon 3. The sequence of subsequent amino acids was unchanged. Exons in other splice variants were omitted in their entirety.

We investigated whether these variants are expressed in other cell types. Figures 2c and d show that all examined cells expressed these splice variants, but in different proportions.

When expression vectors of these splice variants were transfected into NHEKs, full-length transcript as well as Δ4 and Δ4–5, which are variants lacking exon 4 and/or exon 5, were detected in the nuclear fraction, whereas Δ3, the construct lacking exon 3, was detected in the cytoplasmic fraction (Figure 2e).

This study demonstrated the existence of multiple splice variants of IL-33 in several different cell types, with differing expression patterns among different cell types.

The existence of multiple splice variants is known for several cytokines, including IL-2, IL-4, IL-6, IL-7, IL-10, IL-21, and IL-24 (Whitaker *et al.*, 2011). In many of these cases, the protein products of alternatively spliced variants function to modulate the activity of the primary isoform.

A recent study demonstrated the existence of an IL-33 splice variant lacking exon 3 (exon 4 in this study), and showed that the protein possesses constitutive cytokine activity (Hong *et al.*, 2011).

Exon 3 contains a nuclear-localization motif (Carriere *et al.*, 2007), consistent with the observation that recombinant IL-33 that lacks this region was not detected in the nucleus. There was a tendency for the transformed cells to lack certain splice variants, such as those lacking exon 3 or 4, which suggests that the transformation of the cells may affect the pattern of expression. This aspect requires further study.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Topical Application of Thymidine Dinucleotide to Newborn Mice Reduces and Delays Development of UV-Induced Melanomas

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TO THE EDITOR

One major risk factor for cutaneous melanoma is UV exposure. Intense intermittent UV exposure and childhood sunburn are linked epidemiologically with melanoma risk, and in mice neonatal UV exposure promotes development of cutaneous melanoma (Noonan *et al.*, 2001; Kannan *et al.*, 2003). Other evidence that UV contributes to melanomagenesis includes increased risk for populations with extensive intense sun exposure, as well as for fair-skinned (Fitzpatrick's skin type I–II) individuals and patients with xeroderma pigmentosum, who repair photoproducts very poorly (Kraemer *et al.*, 1987; Gilchrest *et al.*, 1999).

We have previously shown that telomere homolog oligonucleotides (collectively called T-oligos), but not complementary or unrelated control oligonucleotides, have multiple anticancer effects (Eller *et al.*, 1997; Puri *et al.*, 2004; Gilchrest and Eller, 2009). T-oligo treatment before UV irradiation accelerates the removal of major UV-induced cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts in cultured cells from newborn and adult donors (Goukassian *et al.*, 2002), murine skin *in vivo* (Goukassian *et al.*, 2004; Arad *et al.*, 2008), and human skin *ex vivo* (Arad *et al.*, 2007). T-oligos have also been shown to cause cell cycle arrest, followed in many malignant cell types by apoptosis (Puri *et al.*, 2004; Gilchrest and Eller, 2009). We have previously shown that treatment with T-oligos (specifically, thymidine dinucleotide (pTT)) during chronic UV irradiation prevents development of squamous cell carcinoma (SCC) in hairless mice (Goukassian *et al.*, 2004) and of basal cell carcinoma (BCC) in Ptch-1$^{+/-}$ mice (Arad *et al.*, 2008). In these models, intermittent topical pTT application enhances DNA repair of CPDs and 8-oxo-2′-deoxyguanosine and decreases mutagenesis, and in tumor nodules it increases apoptosis and decreases proliferation. pTT also strikingly reduces Cox-2 protein expression in UV-irradiated skin (Arad *et al.*, 2008).

Many mutations associated with familial melanoma occur at the CDKN2A locus that encodes two distinct proteins, p16 INK4a and p14 ARF (p19 ARF in mice) (Chudnovsky *et al.*, 2005). Several knockout (KO) and transgenic animal models have been developed to study p16- and p19-dependent molecular mechanisms of melanoma development. Of interest to modeling human melanomagenesis, when p19$^{ARF-/-}$ mice expressing H-ras driven by a tyrosinase promoter (Tyr-Hras/p19KO mice) are UV irradiated on day 2 or 3 after birth, there is a significant increase in melanoma development during early adulthood (Kannan *et al.*, 2003). In this study we evaluated the effect of topical pTT treatment in this model.

Newborn mice were treated topically with a 100 μM solution of pTT (Midland Certified Reagent Company, Midland, TX) or the PG/DMSO (propylene glycol 75%/DMSO 25%) vehicle alone on days 1 and 2, then UV irradiated on day 3 using FS40 sunlamps (10 mJ cm^{-2}) as metered at 285 ± 5 mm, an irradiation protocol known to cause melanomas by week 21 in approximately half the mice (Kannan *et al.*, 2003).

pTT-treated mice began to develop melanomas during week 12, whereas vehicle-treated mice began to develop tumors during week 7; and by week 21, 71% versus 46% of the mice remained tumor free (Figure 1a). All mice were examined weekly and killed if their tumors were ⩾1 cm in diameter or the

Abbreviations: ANOVA, analysis of variance; BCC, basal cell carcinoma; CPD, cyclobutane pyramidine dimer; KO, knockout; pTT, thymidine dinucleotide; PG/DMSO, propylene glycol 75%/DMSO 25%; SCC, squamous cell carcinoma; T-oligo, telomere homolog oligonucleotide

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