Assessment of the relative number of copies of the gene encoding human neutrophil antigen-2a (HNA-2a), *CD177*, and a homologous pseudogene by quantitative real-time PCR

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Human neutrophil antigen-2a (HNA-2a; NB1) is located on the 58–64 kD NB1 glycoprotein (GP) and is encoded by the gene *CD177*. Searches of human genome databases have revealed that a pseudogene highly homologous to exons 4–9 of *CD177* is located adjacent to *CD177* on chromosome 19. The purpose of this study was to document the presence of the pseudogene and determine whether the polymorphic expression of NB1 GP is due to *CD177* gene deletions and duplications. Genomic DNA was isolated from leukocytes of 12 subjects. The number of copies of exon 2 of *CD177,* an exon that is unique to this gene, and the number of copies of exon 9, an exon that is found in both *CD177* and the pseudogene, was assessed with quantitative real-time PCR. The ratio of the number of copies of sequences homologous to *CD177* exon 9 to the number of copies of exon 2 was 1.5 or greater in 7 of the 12 subjects, suggesting that both *CD177* and the homologous pseudogene were present. The ratio of exon 9 to exon 2 in the other 5 subjects ranged from 1 to 1.25, suggesting that the pseudogene was not present in these subjects. However, results of assays were variable and we could not exclude the possibility that all subjects carried the pseudogene. These studies confirmed the presence of the pseudogene homologous to *CD177,* but quantitative real-time PCR was not precise enough to detect *CD177* duplications or deletions. ***Immunohematology*** **2003;19:122–126.**

Key Words: HNA-2a, *CD177* gene, pseudogene, real-time PCR

Human neutrophil antigen-2a (HNA-2a) is located on NB1 glycoprotein (GP) and is encoded by *CD177.*[1–4] The antigen frequency of HNA-2a is 93 percent to 97 percent and HNA-2a is unique in that it is expressed on subpopulations of neutrophils.[5–7] *CD177* belongs to the Ly-6 superfamily. Members of this gene superfamily are characterized by conserved 70- to 80-amino acid domains containing ten cysteine residues. However, genes in this family encode proteins with diverse functions and members of this family show relatively little nucleotide identity. Other genes in this family that are expressed by blood cells include urokinase-type plasminogen activator receptor (*uPAR, CD87*), which is found on leukocytes, and reactive inhibitor of lysis receptor (*CD59*), which is found on both RBCs and leukocytes.

The function of the NB1 GP encoded by *CD177* is not known, but *CD177* mRNA levels are increased in neutrophils from patients with polycythemia vera and from people receiving granulocyte-colony stimulating factor.[8,9] Antibodies to HNA-2a can cause neonatal alloimmune neutropenia, autoimmune neutropenia, TRALI, or delayed recovery of neutrophil counts after marrow transplantation.[10]

CD177 is located on chromosome 19q13.2 and has 9 exons.[11] Searches of the human genome databases suggest that adjacent to *CD177* is a pseudogene that is highly homologous to exons 4 through 9 of *CD177* (Fig. 1).[11] Pseudogenes have sequences related to functional genes, but are unable to code for proteins due to deficiencies that affect translation or transcription. Because of the close proximity and high homology of *CD177* and the pseudogene, human genome database data did not allow the determination of the exact structure of chromosome 19 in the region of *CD177* and the pseudogene. The purpose of this study was to obtain experimental data that

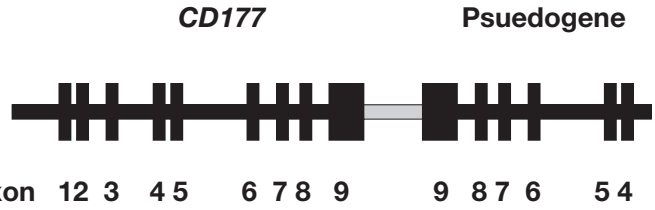

Fig. 1. The proposed structure of *CD177* and a homologous pseudogene in chromosome 19q13.2. The structure of chromosome 19q13.2 was determined using GenBank and Joint Genome Institute databases. The GenBank data in this region of chromosome 19 were incomplete, creating a sequence gap. Public Joint Genome Institute databases allowed Bettinotti and colleagues to predict this structure of 19q13.2.[11]

documented the presence of the pseudogene homologous to *CD177* and, if *CD177* gene duplications, deletions, or both occur, determine whether they are responsible in part for the polymorphic expression of HNA-2a and NB1 glycoprotein.

Quantitative real-time PCR uses PCR and a fluorogenic probe designed to be incorporated in the DNA being amplified.[12] In this assay, relative increases in fluorescent emissions are monitored during PCR, using an analytical thermal cycler. Quantitative real-time PCR is being used to quantitate small amounts of DNA or mRNA.

Neutrophil HNA-1 antigens are located on neutrophil Fc-gamma receptor IIIb (FcγRIIIb) and are encoded by *FCGR3B.*[10] The polymorphic expression of FcγRIIIb is in part due to duplications and deletions of *FCGR3B.* Quantitative real-time PCR has been used to document *FCGR3B* gene duplications[13] and we thought that a similar assay could be used to document the presence of the pseudogene homologous to *CD177.* We used quantitative real-time PCR to compare the numbers of copies of *CD177* and the *CD177* pseudogene.

Methods and Materials

Study design

The number of copies of the *CD177* gene and of the *CD177* pseudogene was assessed among several people, using quantitative real-time PCR. Since the number of copies of genomic *CD177* and of the *CD177* pseudogene was being measured, DNA was isolated from peripheral blood leukocytes. Quantitative real-time PCR was used to measure copies of genomic DNA that was homologous to a part of *CD177* that was shared by both *CD177* and the homologous pseudogene, exon 9, and a part of *CD177* that was not shared by the pseudogene, exon 2. The ratio of the number of copies of the *CD177* exon 9 to *CD177* exon 2 was compared. If each chromosome 19 in a subject contains one copy of the *CD177* gene and one copy of the pseudogene, then the ratio of the number of copies of exon 9 to exon 2 is 2.0 (Table 1). If a pseudogene is present, but *CD177* is not, then the ratio of exon 9 to exon 2 is infinity. If *CD177* is present, but the pseudogene is not, then the ratio is 1.0. If a *CD177* gene duplication is present, the ratio is 1.5. The ratio will be less than 1.5 if multiple duplications are present. If a *CD177* pseudogene duplication is present, then the ratio of exon 9 to exon 2 is 3.0. The ratio is greater than 3.0 if multiple duplications are present (Table 1).

Table 1. Expected outcomes of comparisons of copies of genomic sequence homologous to exons 2 and 9 of *CD177* in people with various genotypes

	Copies of each in chromosome 19				(Exon 9 copies)/
Genotype*	Gene	Pseudogene	Exon 2	Exon 9	(Exon 2 copies)
1 *CD177* + 1 *CD177* pseudogene	1	1	1	2	2.0
CD177 deletion	0	1	0	1	infinity
CD177 duplication	2	1	2	3	1.5
CD177 pseudogene deletion	1	0	1	1	1.0
CD177 pseudogene duplication	1	2	1	3	3.0

*Assuming that the subjects are homozygous

Quantitative real-time PCR

Leukocyte DNA was isolated from 1.0 mL of whole blood collected in ACD using a kit according to the manufacturer's instructions (Puregene, Gentra Systems, Minneapolis, MN). One microliter leukocyte DNA at a concentration of 100 µg/mL was used as a template to measure genomic sequences homologous to exons 2 and 9 of *CD177* by quantitative real-time PCR using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer of Foster City, CA). Quantitative real-time PCR results were reported as the number of copies of exon 9 divided by the number of copies of exon 2.

Primers and TaqMan probes were designed to produce amplicons < 150 bp, enhancing the efficiency of PCR amplification (Biosource International, Camarillo, CA) (Table 2). TaqMan probes were labeled at the 5' end with the reporter dye molecule FAM (6-carboxyfluorescein; emission λ_{max} = 518 nm) and at the 3' end with the quencher dye molecule TAMRA (6

Table 2. Primers and probes used to analyze by quantitative real-time PCR genomic sequences homologous to exons 2 and 9 of *CD177*

Exon	Type	Sequence	Amplicon Size (bp)
2	Forward primer-ex 2	5'GGCAATGGACCCCTAAGAACA 3'	74
	Reverse primer-ex 2	5'GCTCTCAATGAGCATCAACGTG 3'	
	Probe-ex 2	5'CAGCTGCGACAGCGGCTTGG 3'	
9	Forward primer-ex 9	5'GCCCAACCTTCCAGCTTCTT 3'	79
	Reverse primer-ex 9	5'GCTGCACATCACGCTTCTCAC 3'	
	Probe ex 9	5'TTGAACCACACCAGACAAATCGGG 3'	

carboxytetramethylrhodamine; emission λ_{max} = 582 nm) (Table 2). The *CD177* coding region was isolated and used as a standard curve. The coding region of *CD177* was amplified using sequence-specific primers from human fetal liver total RNA (Stratagene, La Jolla, CA) and the amplicon was cloned using a Topo TA cloning kit (Invitrogen Corporation, Huntsville, AL). DNA from several of the clones was analyzed for the correct size insert by digestion with Eco R1 and analyzed further by sequencing using sequence-specific primers and a cycle-sequencing kit (Big Dye Terminator, Perkin-Elmer Applied Biosystems, Inc., Foster City, CA). The sequencing reaction products were purified using the DyeEx Spin Kit (Qiagen Inc., Valencia, CA) according to manufacturer's instructions and the reactions were analyzed on a genetic analyzer (ABI Prism 377, Perkin-Elmer). The *CD177* coding sequence was purified and quantified by spectrophotometry (OD260). The number of DNA copies was calculated using the molecular weight of each gene amplicon. Serial dilutions of the amplified gene at known concentrations were tested by quantitative real-time PCR.

Quantitative real-time PCR reactions of DNA specimens and standards were conducted in a total volume of 25 µL with 1 × TaqMan Master Mix (Perkin-Elmer) and primers and probes at optimized concentrations (primers 10,000 nM and probes 12,000 nM). Thermal cycler parameters were 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles involving denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. Real-time monitoring of fluorescent emission from the cleavage of sequence-specific probes by the nuclease activity of Taq polymerase allowed definition of the threshold cycle number during the exponential phase of amplification.

Standard curves of the threshold cycle number versus the log of the number of copies of genes were generated for exon 2 and exon 9. The reactions were found to have excellent PCR amplification efficiency (90–100%; 100% indicates that after each cycle the amount of template is doubled) as determined by the slope of the standard curves. Linear regression analysis showed that r^2 for all standard curves was > 0.98.

For each sample of DNA tested the threshold cycle number was measured with exon 2 primers and probes and exon 9 primers and probes. The standard curves were used to extrapolate the number of copies of exon 2 and exon 9. All PCR assays were performed in quadruplicate and reported as the average.

Results

Comparison of CD177 and CD177 pseudogene copy numbers

In 12 subjects quantitative real-time PCR was used to compare the number of copies of genomic sequences that were homologous to exons 2 and 9 of *CD177.* In all subjects the number of copies of exon 9 was greater than or approximately equal to the number of copies of exon 2. The ratio of copies of exon 9 to copies of exon 2 varied from 0.98 for subject 11 to 3.33 for subject three (Table 3). In seven of 12 subjects the number of copies of exon 9 was greater than the number of copies of exon 2: exon 9 to exon 2 ratios of 1.45 or greater. These results suggest that both *CD177* and the pseudogene were present in these seven subjects. In the other five subjects, the number of copies of exon 9 was approximately equal to the number of copies of exon 2: exon 9 to exon 2 ratios from 0.98 to 1.24. These results suggest that for these five subjects, *CD177* was present, but the homologous pseudogene was not.

Table 3. Comparison of the ratio of the numbers of genomic sequences homologous to exons 2 and 9 of *CD177* as determined by quantitative real-time PCR

Donor	Copies Exon 9/Copies Exon 2	Interpretation
1	1.13	No pseudogene
2	1.24	No pseudogene
3	3.33	Gene and pseudogene
4	2.54	Gene and pseudogene
5	2.38	Gene and pseudogene
6	1.20	No pseudogene
7	1.45	Gene and pseudogene
8	1.86	Gene and pseudogene
9	2.56	Gene and pseudogene
10	2.20	Gene and pseudogene
11	0.98	No pseudogene
12	1.12	No pseudogene

Reproducibility of results

To compare the reproducibility of the quantitative real-time PCR assay in five subjects, comparisons of genomic sequences homologous to *CD177* sequences in exon 2 and exon 9 were performed on two separate occasions. In three of the five subjects the results of the first and second tests were very similar, but in two subjects the ratio of the number of copies of exon 9 to exon 2 varied markedly (Fig. 2).

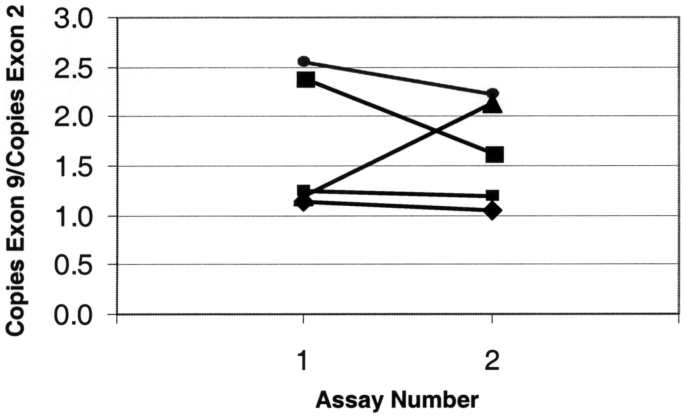

Fig. 2. The results of repeat analysis of the ratio of genomic sequences homologous to exon 9 and exon 2 of *CD177*. In five subjects the ratio of the quantities of sequences in leukocyte DNA homologous to exon 9 and exon 2 of *CD177* was measured on two separate occasions using quantitative real-time PCR.

Discussion

These results provide experimental support of information in the human genome database that suggested that a pseudogene that is highly homologous to *CD177* is present in the human genome. Our experimental data suggest that both *CD177* and the homologous pseudogene were present in seven of 12 subjects tested. However, the large degree of variability inherent in the assay makes our results difficult to interpret and we cannot exclude the possibility that all subjects carry the pseudogene.

We had hoped to compare the size of the neutrophil population that expressed NB1 GP with a measurement of the number of copies of *CD177* relative to the number of copies of the pseudogene. However, the quantitative real-time PCR assay was not precise enough to determine the presence of duplications or deletions of *CD177* or the homologous pseudogene. Other investigators have reported that the reproducibility of the threshold cycle number measured by quantitative real-time PCR is 2 percent to 5 percent.[14] However, the threshold cycle number is linearly related to the log of the gene copy number. As a result the variability of the number of gene copies is greater and can be as high as 12 percent. Since we were reporting one measure divided by another, the potential for variability is even greater. Our results are consistent with those of Gittinger and colleagues, who found variations in expected gene copy number and actual gene copy number were as great as 50 percent.[13]

Wolff and colleagues have used quantitative real-time PCR to compare neutrophil *CD177* mRNA copy numbers and the proportion of neutrophils expressing NB1 GP.[9] They found that people with a larger population of neutrophils expressing NB1 GP had greater quantities of *CD177* mRNA in their neutrophils, but the quantities of *CD177* mRNA varied more than 100-fold among individuals. In addition, Temerinac and colleagues have found that *CD177* mRNA levels are greater in neutrophils from people with polycythemia rubra vera and in some people with essential thrombocytosis.[8] However, in patients with polycythemia rubra vera *CD177* mRNA levels were severalfold greater than in healthy subjects.[8,15,16] These large variations in *CD177* mRNA levels make quantitative real-time PCR a useful tool in assessing *CD177* mRNA levels.

In conclusion, we confirmed that the genome carries a pseudogene homologous to *CD177*. While quantitative real-time PCR is important in studying and comparing *CD177* mRNA levels, it was not helpful in measuring and comparing genomic *CD177* copy numbers.

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