PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :		(11) International Publication Number:	**WO 00/32826**
C12Q 1/70, C12P 19/34, C07H 21/04, C12N 5/00	**A1**	(43) International Publication Date:	8 June 2000 (08.06.00)

(21) International Application Number: PCT/US99/28403

(22) International Filing Date: 30 November 1999 (30.11.99)

(30) Priority Data:
60/110,268   30 November 1998 (30.11.98)   US

(71) Applicant *(for all designated States except US)*: DREXEL UNIVERSITY [US/US]; 32nd and Chestnut Streets, Philadelphia, PA 19104 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: SPOTILA, Loretta, D. [US/US]; 161 Merion Avenue, Haddonfield, NJ 08033 (US).

(74) Agents: LICATA, Jane, Massey et al.; Law Offices of Jane Massey Licata, 66 E. Main Street, Marlton, NJ 08053 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS AND KITS FOR IDENTIFYING INDIVIDUALS AT RISK OF DEVELOPING OSTEOPOROSIS

(57) Abstract

Methods and kits for identifying individuals at risk of developing osteoporosis are provided. These methods and kits are based on detecting the presence of polymorphisms in the tumor necrosis factor alpha 2 receptor gene associated with low bone density and the risk of developing osteoporosis.

METHODS AND KITS FOR IDENTIFYING INDIVIDUALS AT RISK
OF DEVELOPING OSTEOPOROSIS

INTRODUCTION

5   This invention was made in the course of research
sponsored by the National Institutes of Health. The U.S.
Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

Osteoporosis is a disabling condition characterized by
10 low bone density and fragile bones. The term
"osteoporosis", encompasses diseases of many different
etiologies that all result in a reduction in the mass of
bone per unit volume. Osteoporosis can be seen without
association with other disease (idiopathic) and also in
15 conjunction with other diseases (e.g., rheumatoid
arthritis, diabetes, calcium deficiency). The decreased
bone mass characteristic of the disease is not due to a
decrease in the ratio of mineral to organic phase of bone,
nor to any known abnormality in the mineral or organic
20 matter of bone. Histological changes seen include a
decrease in cortical thickness and a decrease in the number
and size of the trabeculae of cancellous bone.
Osteoporosis is the most common of all metabolic bone
diseases and is seen commonly in the elderly.

25   Since bone mass is decreased in osteoporosis, the rate
of bone resorption exceeds that of bone formation. There
is a normal age-related loss of bone mass in adults.
However, this age-related loss of bone begins earlier and
proceeds more rapidly in women. The reasons for the age-
30 related bone loss are not known, although risk factors
identified have included race (white women being at
greatest risk), sex (females), sedentary lifestyle, and
less muscular development. Other factors associated with

-2-

risk of developing osteoporosis include early menopause,
smoking, and excessive alcohol consumption.

Diagnosis of osteoporosis is most often done in
conjunction with a study of bone density by radiography.

5 Although clinical laboratory tests such as levels of
calcium and phosphorus in blood can be examined, these
measures are usually normal in osteoporotic patients. Only
about 20% of postmenopausal women with osteoporosis exhibit
hypercalciuria, or increased excretion of calcium in urine.

10 Therefore, such laboratory findings are not indicative of
the presence of disease, and clearly would not be
indicative of risk of developing disease. To date, the
prediction of risk of developing disease relies on family
history of the disease. However, no genetic test is

15 currently available to screen individuals.

The ethnic differences shown to exist in the
propensity to develop osteoporosis have led researchers to
believe that genetic factors play a dominant role in the
etiology of this disease. Several genes have been shown to

20 be associated with low bone density and research has
focused on identifying those genes that may act as markers
of disease. Common allelic variations of the vitamin D
receptor gene have been found to be associated with
decreased bone density in certain populations, including

25 premenopausal women and young girls (Wood, R.J. and Fleet,
J.C. Ann. Rev. Nutrit. 1998 18:233-258). Bone mineral
density has also been associated with genetic variation in
the estrogen receptor gene, both by itself and in
conjunction with variations in the vitamin D receptor gene

30 (Willing et al. J. Bone Min. Res. 1998 13:695-705). In
Japanese women, the HLA-A*24-B*07-DRB*01 halotype has been
linked to low peak bone mass (Tsuji et al. Hum. Immunol.
1998 59:243-249). A variant of the gene encoding
transforming growth factor-beta 1 has also been associated

35 with low bone mass in osteoporotic women and with low bone

-3-

mass and increased bone turnover in normal women (Langdahl et al. *Bone* 1997 20:289-294). A polymorphism of the COL1A1 gene has been identified as a potential marker for low bone mass and vertebral fracture in women (Grant et al. *Nat.*

5 *Genet.* 1996 14:203-205). However, none of these studies has examined the relationship between tumor necrosis factor alpha receptor 2 gene and osteoporosis or bone mass in women.

Devoto et al. (*Eur. J. Hum. Genet.* 1998 6:151-157)

10 determined that there was a gene or genes on chromosome 1 of humans that was linked to low bone density. However, there are many potential genes on this chromosome that could be involved in the etiology of low bone density.

It has now been found that individuals at risk of

15 developing osteoporosis can be identified by assessing the genotype of the tumor necrosis factor alpha receptor gene in a sample of DNA obtained form the individual. A polymorphism in the gene on chromosome 1 for tumor necrosis factor alpha receptor 2 has now been shown to be associated

20 with low bone density.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a method of screening individuals for risk of developing osteoporosis. This method is based on assessment of the

25 genotype of the tumor necrosis factor alpha gene in a DNA sample obtained from the individual. The presence of polymorphisms at nucleotides 593, 598 and 620 in exon 10 of the tumor necrosis factor alpha 2 receptor gene is associated with low bone density.

30 Another object of the present invention is to provide a kit for screening individuals for risk of developing osteoporosis which comprises a means for assessing the genotype of the tumor necrosis factor alpha 2 receptor gene in an individual.

-4-

DETAILED DESCRIPTION OF THE INVENTION

In the present invention a method is provided for
screening individuals for the presence of a polymorphism in
the tumor necrosis factor alpha receptor 2 (TNFR2) gene.
5 The presence of this genotype in individuals has now been
shown to be associated with low bone density and the risk
of developing osteoporosis. The association between TNFR2
gene and low bone density was based on the observation that
there is a polymorphism in this gene at a particular region
10 of the gene sequence. This polymorphism was identified by
Kaufman et al. (*Hum. Genet.* 1994 94:418-422), however, no
characterization of its nature or examination of the
relationship with bone density was disclosed. In the
present invention, a relationship of this polymorphism with
15 bone density has now been identified.

In these experiments, two polymorphic sites within the
TNFR-2 gene, including a microsatellite repeat within
intron 4 and a three nucleotide variation in the
3'untranslated region of the gene were assayed in three
20 groups of individuals. Test group 1 was comprised of
individuals from families used for linkage analysis of the
low BMD (mean bone density) trait. Test group 2 was made
up of unrelated individuals from an osteoporosis clinic.
The third group was made up of unrelated individuals from a
25 general clinic population with no testing for or knowledge
of their bone status. This third group was used as
reference for allele frequency comparisons. The
composition and mean bone density Z-scores for test groups
1 and 2 are shown in Table 1.

-5-

Table 1:   Results of ANOVA among the males and females
           comprising the two test groups for age, body mass
           index and bone density

	GROUP 1		GROUP 2		P Value	F ratio (df)
	Male	Female	Male	Female		
Number	22	44	12	81		
Age (yrs ± SE)	56.2 (2.5)	57.3 (1.9)	43.7 (3.8)	52.6 (1.5)	0.011	3.846 (3,153)
Body Mass Index (±SE)	28.4 (1.3)	25.9 (0.9)	25.0 (1.7)	24.8 (0.6)	0.084	2.259 (3,149)
Mean BMD Z-score (±SE)						
L2-L4	-0.56 (0.38)	-1.17 (0.21)	-1.02 (0.49)	-0.99 (0.14)	0.442	0.901 (3,155)
Femoral neck	-0.50 (0.27)	-0.74 (0.13)	-0.73 (0.39)	-0.74 (0.09)	0.790	0.348 (3,150)
Trochanter	0.04 (0.31)	-0.64 (0.16)	-0.84 (0.30)	-0.59 (0.11)	0.054	2.611 (3,146)
Ward's triangle	-0.21 (0.22)	-0.76 (0.16)	-0.61 (0.45)	-0.70 (0.11)	0.232	1.446 (3,146)

Only age was significantly different among the groups
(p=0.011), due to the younger mean age of the males in the
second group. However, ANOVA of age as the dependent
variable with genotype for the two polymorphisms as class
variable indicated that age was unaffected by genotype.
Therefore the results of genotype testing in the two groups
were pooled for subsequent analyses.

The microsatellite repeat within intron 4 of the TNFR2
gene has been reported previously to have 5 alleles
(Beltinger et al. *Genomics* 1996 35:94-100). In these test
groups, 6 alleles were observed with a statistically
significant difference in the frequency of distribution
between the two groups (p<0.025, Table 2). A total of 18
different genotypes would be expected from 6 alleles
assuming Hardy-Weinberg equilibrium. The observed
frequency distribution of genotypes of the intron 4
microsatellite repeat for both the test and control groups

-6-

did not differ significantly from expected (Test group Chi-square=20.54, df=19, N.S.; control group Chi-square=10.08, df=19, N.S.). Comparison of the genotype distributions between the test and control groups was also not
5 significant (Chi-square=22.46, df=19, N.S.).

The polymorphism within the 3'UTR of the TNFR2 gene was previously characterized as having 4 alleles that were identified by different banding patterns under electrophoretic conditions that detect single strand
10 conformation differences (Kaufman et al. *Hum. Genet.* 1994 94:418-422; White et al. *Genes, Chrom., Canc.* 1993 7:102-108; and Kaufman et al. *Hum. Molec. Genet.* 1993 2:824). Using a different conformation sensitive gel technique, 5 alleles have now been resolved, each as a single band
15 (Table 2). The allele frequency distribution was compared between the test and control groups, with no statistically significant differences. The frequency distribution of the 15 genotypes was in Hardy-Weinberg equilibrium in both groups (Test group Chi-square= 8.44, df=14, N.S.; Control
20 group Chi-square=22.57, df=14, N.S.). However, comparison of the genotype distributions for the 3'UTR polymorphism between the test and control groups was statistically significant (Chi-square=48.70, df=14, p<0.001).

Table 2:   Allele frequency distribution for the two TNFR2
25   polymorphisms in test group (n=159) compared with
              control group (n=141)

Allele	Intron 4 Polymorphism*		UTR Polymorphism**	
	Test group	Controls	Test group	Controls
1	0.195	0.156	0.059	0.038
2	0.037	0.080	0.229	0.240
3	0.573	0.551	0.154	0.137

4	0.186	0.193	0.374	0.427
5	0.006	0.004	0.182	0.156
6	0.003	0.015	NA***	NA

*Chi Square = 14.74, df=5, p<0.025

5   **Chi Square = 8.24, df=4, p<0.10

***NA=Not applicable

The nature of the UTR polymorphism was established by sequencing of PCR product from individuals who had been scored as homozygous for alleles 2, 3, 4 and 5 or

10   heterozygous for combination of allele 1 and the other four alleles. No 1/1 homozygotes were observed. The polymorphism is actually a combination of three polymorphic sites within a span of 28 nucleotides, affecting nucleotides 593, 598 and 620 of exon 10 of the TNFR2 gene

15   (Genbank Accession #U52165; SEQ ID NO:1). The five observed alleles are defined as summarized in Table 3.

Table 3:   Alleles of the TNFR2 UTR polymorphism as defined
            by nucleotide composition at three variable
            sites. Numbering is based on Genbank Accession

20   #U52165

Alleles	Nucleotide 593	Nucleotide 598	Nucleotide 620
1	A	G	T
2	A	T	T
3	G	T	C
4	G	T	T
5	A	T	C

Analysis of variance (ANOVA) was performed on BMD Z-scores at the spine, femoral neck, trochanter and Ward's triangle with respect to genotype at each polymorphic site

30   (Table 4).

-8-

Table 4:   Results of ANOVA of BMD Z-scores with genotype
           for each of the two TNFR2 polymorphisms as the
           grouping variable in the study group (n=159)

	TNFR2 Polymorphism			
	Intron 4 Microsatellite		3'UTR	
	P value	F ratio (df)	P value	F ratio (df)
L2-L4	0.044	1.937 (10,146)	0.170	1.386 (13,145)
Femoral Neck	0.120	1.574 (10,141)	0.140	1.459 (13,140)
Trochanter	0.199	1.373 (10,137)	0.127	1.494 (13,136)
Ward's triangle	0.199	1.372 (10,137)	0.149	1.439 (13,136)

No statistically significant relationship was observed
(i.e. $p<0.025$). Analysis of the genotype data using Tukey-
Kramer post hoc pair-wise comparisons indicated that the
mean Z-score for spinal BMD of individuals having genotype
1/2 for the UTR polymorphism was significantly different
from those with genotype 2/2. Analysis of genotype data
for the CA polymorphism by the Tukey-Kramer test suggested
no significant differences. However, when the UTR
genotypes were placed in order with respect to mean spinal
BMD Z-scores, it was found that the lowest mean spinal BMD
occurred in the genotype groups that had allele 1, the
rarest allele (Table 5).

Table 5:   Genotypes for the TNFR2 UTR polymorphism with
           frequency and mean spinal or femoral neck BMD Z-
           scores for the study group

GENOTYPE	FREQUENCY	SPINAL BMD Z-SCORE* (Mean ± SE)	FEMORAL NECK BMD Z-SCORE** (Mean ± SE)
1/2	5	-2.814 (0.633)	-0.727 (0.469)

1/5	4	-1.718 (0.708)	-1.445 (0.469)
1/3	2	-1.345 (1.00)	-1.620 (0.663)
1/4	8	-1.316 (0.500)	-0.454 (0.354)
3/4	21	-1.225 (0.309)	-0.920 (0.215)
2/3	7	-1.218 (0.535)	-0.494 (0.354)
2/4	26	-1.124 (0.277)	-0.777 (0.184)
2/5	13	-0.980 (0.393)	-0.342 (0.260)
3/3	6	-0.778 (0.578)	-0.458 (0.419)
4/4	23	-0.776 (0.295)	-0.868 (0.195)
3/5	7	-0.750 (0.535)	-1.108 (0.354)
5/5	8	-0.719 (0.500)	-1.130 (0.331)
4/5	18	-0.684 (0.334)	-0.508 (0.221)
2/2	11	-0.023 (0.427)	-0.022 (0.282)
Total	159		

*ANOVA, p=0.17, R^2=0.11
**ANOVA, p=0.14, R^2=0.12

The mean femoral neck BMD Z-scores are shown for
comparison, and indicate a similar trend in that the two
lowest mean Z-scores occur in groups having allele 1.

Thus, these analysis show that the frequency of the
rarest allele of this gene is associated with low bone
density. Further, this association between the genotype of
TNFR2 and bone density is statistically significant
(p=0.008). Accordingly, assessment of the genotype of the
TNFR2 gene of an individual is useful in identifying
individuals with low bone density at risk for osteoporosis.

In the method of the present invention, a DNA sample
is obtained from an individual. The genotype of the TNFR2
gene is then assessed to determine if the individual is at
risk for developing osteoporosis. For example, detection
of a polymorphisms at nucleotides 593, 598 and 620 of exon
10 of the TNFR2 gene (SEQ ID NO:1) is indicative of an
individual at risk of developing osteoporosis.

-10-

The present invention also relates to diagnostic kits comprising a means for identifying individuals at risk of developing osteoporosis based on assessment of the genotype of the TNFR2 gene. In a preferred embodiment, such means

5 are capable of detecting polymorphisms at nucleotides 593, 598 and 620 of exon 10 of the TNFR2 gene (SEQ ID NO:1). Such means can be developed routinely by those skilled in the art based upon known techniques for genotype screening such as PCR and gel electrophoresis. In one embodiment,

10 the kit comprises primers such as exemplified by SEQ ID NO: 4 and SEQ ID NO:5 described in Example 3 herein. However, as will be obvious to those of skill in the art upon this disclosure, additional means useful in assessing the TNFR2 genotype of an individual can also be used. In a preferred

15 embodiment, the kits of the present invention further comprise a blood collection device for obtaining a blood sample from an individual as the source of DNA for genotyping. Other sources of DNA obtained from a patient which can be genotyped via the kits and methods of the

20 present invention include both tissue samples and other biological fluids.

The following non-limiting examples are provided to further illustrate certain aspects of the instant invention.

Using the method and/or kits of the present invention, one of skill can screen individuals known to have a family history of low bone density and/or individuals with

25 identified risk factors for osteoporosis and low bone density including, but not limited to, post-menopausal women and the elderly. Via these methods and kits, individuals having polymorphisms in the TNFR2 gene, and in particular polymorphisms detected at nucleotide 593, 598

30 and 620 of exon 10 of the TNFR2 gene (SEQ ID NO:1), can be identified as being at risk of developing osteoporosis.

The following non-limiting examples are provided to further illustrate certain aspects of the instant invention.

-11-

EXAMPLES

Example 1:   Subjects

The first group of test subjects was composed of 66
individuals unrelated to each other. These individuals

5   were either the founders of or had married into families
with low BMD. The second test group of 93 individuals were
also unrelated to each other and to the first group and had
been referred to the McGill Bone Center by physicians for
evaluation of bone status. The third group was a random

10   general clinic population (n=141), that served as a control
for allele frequency distribution in these studies.

Blood was collected from participants and DNA was
isolated by standard methods. Bone densitometry was
performed on individuals in the first two groups in

15   accordance with procedures described by Devoto et al. *Eur.
J. Hum. Genet.* 1998 6:151-157 and Spotila et al. *Calcif.
Tissue Int.* 1996 59:235-237.

Example 2:   Microsatellite Repeat in Intron 4

The PCR primers for detection of the $(CA)_{16}$

20   microsatellite repeat described by Beltinger et al.
(*Genomics* 1996 35:94-100) were TNFR2-CAF:5'-
GTGATCTGCAAGATGAACTCAC-3' (SEQ ID NO:2) and TNFR2-CAR:5'-
ACACCACGTCTGATGTTTCA-3'(SEQ ID NO:3). PCR conditions were
denaturation at 94.5°C for 3 minutes; followed by 5 cycles

25   of 94.5°C for 30 seconds, 65.0°C for 30 seconds, 72°C for 30
seconds, followed by 23 cycles of 94.5°C for 30 seconds,
55.0°C for 30 seconds, 72.0°C for 30 seconds; followed by
72.0°C for 5 minutes in a Perkin Elmer Thermocycler 9600.
The primer TNFR2-CAF was labeled with γ-^{32}P-ATP using T4

30   polynucleotide kinase. Approximately 100-300 ng of DNA
template was used with reaction components under conditions
recommended by the manufacturer (Applied Biosystems).

Example 3:   Polymorphism of 3' Untranslated Region

PCR primers for detection of the polymorphism within

-12-

the 3'UTR of TNFR2 as described by Kaufman et al. (Hum. Genet. 1994 94:418-422) were TNFR2-7:5'-AGGACTCTGAGGCTCTTTCT-3' (SEQ ID NO:4) and TNFR2-8:5'-TCACAGAGAGTCAGGGACTT-3' (SEQ ID NO: 5). PCR conditions

5   were denaturation at 94.5°C for 3 minutes; followed by 28 cycles of 94.5°C for 30 seconds, 62.0°C for 30 seconds, 72.0°C for 30 seconds; followed by 72.0°C for 5 minutes. Reagents and instrumentation were the same as described for Example 2.

10   Using conformation sensitive gel electrophoresis (CSGE), 5 differentially migrating fragments of this PCR product were distinguished. Gel conditions and product suppliers were similar to those described by Ganguly et al. (*Proc. Natl Acad. Sci. USA* 1996 93:13721-13725) with the

15 following modifications. Tris-taurine-EDTA running buffer was made as a 20X stock with 1.78 M Tris, 0.58 M Taurine, 4.0 mM EDTA, pH 9.0 and used at 0.5X. Formamide was deionized in batches and frozen in aliquots sufficient for a single gel. Sample loading buffer was prepared each time

20 with 4 parts deionized formamide and 1 part loading buffer (5X=0.1% xylene cyanol, 0.1% bromophenol blue, 10 mM EDTA, 95% formamide). Standard sequencing gel plates and apparatus were used with 0.4 mm spacers. Samples were mixed with an equal volume of sample loading buffer,

25 denatured for 5 minutes at 95°C, transferred to ice and 1.5 μl was immediately loaded. Gels were run at 350 V at room temperature for 16 to 18 hours, then dried and exposed to film. Resolution of 5 alleles was possible only when these conditions were adhered to.

30 **Example 4:   Sequence Analysis**

        Selected PCR fragments were sequenced according to manufacturer's recommendations using ABI 377 instrumentation and Big-Dye chemistry (Applied Biosystems, Inc. Foster City, CA) in order to determine the basis for

35 the 5 alleles.

-13-

Example 5:   Statistical Analysis

The two test groups were compared by ANOVA for age, body mass index and BMD Z-score at the spine, femoral neck, trochanter and Ward's triangle, and then combined for
5   additional analyses. The frequency distribution of alleles for the two polymorphisms within the TNFR2 gene was analyzed by Chi Square. Association of bone density at the spine (L2-L4), femoral neck, trochanter and Ward's triangle with genotype for each of the two polymorphisms was
10 evaluated by ANOVA. The BMD Z-scores at each of the anatomical sites were used as the independent variable since they were already corrected for age, gender and weight. Statistical significance was achieved at $p < 0.025$, because of multiple testing. Pairwise comparisons of mean
15 BMD Z-scores for the different genotypes were made for all pairs using the Tukey-Kramer post hoc test. Power analysis was performed to determine the lowest sample number required to observe a significant ($p = 0.025$) effect of genotype on BMD. The statistical software package employed
20 was JMP (Version 3.1, SAS Institute, Inc. Cary, N.C.).

-14-

What is claimed is:

1.   A method for identifying individuals at risk of developing osteoporosis comprising assessing the genotype of tumor necrosis factor alpha 2 receptor gene in a sample

5   of DNA from an individual.

2.   The method of claim 1 wherein the genotype comprising a polymorphism at nucleotides 593, 598, and 620 in exon 10 of tumor necrosis factor alpha 2 receptor gene (SEQ ID NO:1) is indicative of the individual being at risk

10   for developing osteoporosis.

3.   A kit for identifying individuals at risk of developing osteoporosis comprising a means for assessing the genotype of tumor necrosis factor alpha 2 receptor gene in an individual.

15   4.   The kit of claim 3 wherein the means detects polymorphisms at nucleotides 593, 598 and 620 in exon 10 of tumor necrosis factor alpha 2 receptor gene (SEQ ID NO:1) which are indicative of the individual being at risk for developing osteoporosis.

SEQUENCE LISTING

<110> Spotila, Loretta D.
      Drexel University

<120> Methods and Kits for Identifying Individuals at Risk of
      Developing Osteoporosis

<130> DRE-0019

<140>
<141>

<160> 5

<170> PatentIn Ver. 2.0

<210> 1
<211> 2613
<212> DNA
<213> Homo sapiens

<400> 1
tcttggtctc ggctcctggc ccagtgctct ttcccatgtg tctgaatctg catcttgggc 60
aggggtccct gggccccact cctggacccc cggactgacc cccacccccat cttgtgctta 120
gcagattctt cccctggtgg ccatgggacc caggtcaatg tcacctgcat cgtgaacgtc 180
tgtagcagct ctgaccacag ctcacagtgc tcctcccaag ccagctccac aatgggagac 240
acagattcca gcccctcgga gtccccgaag gacgagcagg tccccttctc caaggaggaa 300
tgtgcctttc ggtcacagct ggagacgcca gagaccctgc tggggagcac cgaagagaag 360
cccctgcccc ttggagtgcc tgatgctggg atgaagccca gttaaccagg ccggtgtggg 420
ctgtgtcgta gccaaggtgg gctgagccct ggcaggatga ccctgcgaag gggccctggt 480
ccttccaggc ccccaccact aggactctga ggctctttct gggccaagtt cctctagtgc 540
cctccacagc cgcagcctcc ctctgacctg caggccaaga gcagaggcag cgagttgggg 600
aaagcctctg ctgccatggt gtgtccctct cggaaggctg gctgggcatg gacgttcggg 660
gcatgctggg gcaagtccct gactctctgt gacctgcccc gcccagctgc acctgccagc 720
ctggcttctg gagcccttgg gtttttttgtt tgtttgtttg tttgtttgtt tgtttctccc 780
cctgggctct gcccagctct ggcttccaga aaaccccagc atccttttct gcagaggggc 840
tttctggaga ggagggatgc tgcctgagtc acccatgaag acaggacagt gcttcagcct 900
gaggctgaga ctgcgggatg gtcctggggc tctgtgtagg gaggaggtgg cagccctgta 960
gggaacgggg tccttcaagt tagctcagga ggcttggaaa gcatcacctc aggccaggtg 1020
cagtggctca cgcctatgat cccagcactt gggaggctg aggcgggtgg atcacctgag 1080
gttaggagtt cgagaccagc ctggccaaca tggtaaaacc ccatctctac taaaaataca 1140
gaaattagcc gggcgtggtg gcgggcacct atagtcccag ctactcagaa gcctgaggct 1200
gggaaatcgt ttgaacccgg gaagcggagg ttgcagggag ccgagatcac gccactgcac 1260
tccagcctgg gcgacagagc gagagtctgt ctcaaaagaa aaaaaaaaa gcaccgcctc 1320
caaatgctaa cttgtccttt tgtaccatgg tgtgaaagtc agatgcccag agggcccagg 1380
caggccacca tattcagtgc tgtggcctgg gcaagataac gcacttctaa ctagaaatct 1440
gccaattttt taaaaaagta agtaccactc aggccaacaa gccaacgaca aagccaaact 1500

1

```
ctgccagcca catccaaccc cccacctgcc atttgcaccc tccgccttca ctccggtgtg 1560
cctgcagccc cgcgcctcct tccttgctgt cctaggccac accatctcct ttcagggaat 1620
ttcaggaact agagatgact gagtcctcgt agccatctct ctactcctac ctcagcctag 1680
accctcctcc tcccccagag gggtgggttc ctcttcccca ctccccacct tcaattcctg 1740
ggccccaaac gggctgccct gccactttgg tacatggcca gtgtgatccc aagtgccagt 1800
cttgtgtctg cgtctgtgtt gcgtgtcgtg ggtgtgtgta gccaaggtcg gtaagttgaa 1860
tggcctgcct tgaagccact gaagctggga ttcctcccca ttagagtcag ccttcccccct 1920
cccagggcca gggccctgca gaggggaaac cagtgtagcc ttgcccggat tctgggagga 1980
agcaggttga ggggctcctg gaaaggctca gtctcaggag catggggata aaggagaagg 2040
catgaaattg tctagcagag caggggcagg gtgataaatt gttgataaat tccactggac 2100
ttgagcttgg cagctgaact attggagggt gggagagccc agccattacc atggagacaa 2160
gaagggtttt ccaccctgga atcaagatgt cagactggct ggctgcagtg acgtgcacct 2220
gtactcagga ggctgagggg aggatcactg gagcccagga gtttgaggct gcagcgagct 2280
atgatcgcgc cactacactc cagcctgagc aacagagtga gaccctgtct cttaaagaaa 2340
aaaaaagtca gactgctggg actggccagg tttctgccca cattggaccc acatgaggac 2400
atgatggagc gcacctgccc cctggtggac agtcctggga gaacctcagg cttccttggc 2460
atcacagggc agagccggga agcgatgaat ttggagactc tgtggggcct tggttcccctt 2520
gtgtgtgtgt gttgatccca agacaatgaa agtttgcact gtatgctgga cggcattcct 2580
gcttatcaat aaacctgttt gttttaaaaa aaa                                  2613
```

```
<210> 2
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic

<400> 2
gtgatctgca agatgaactc ac                                               22

<210> 3
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:   Synthetic

<400> 3
acaccacgtc tgatgtttca                                                  20

<210> 4
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
```

<223> Description of Artificial Sequence: Synthetic

<400> 4
aggactctga ggctctttct   20

<210> 5
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic

<400> 5
tcacagagag tcagggactt   20

INTERNATIONAL SEARCH REPORT	International application No.
	PCT/US99/28403

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/70; C12P 19/34; C07H 21/04; C12N 5/00
US CL : 435/6, 91.1, 372; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 372; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, EAST, WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	BELTINGER et al. Physical mapping and genomic structure of the human *TNFR2* gene. Genomics. 1996, Vol. 35, No. 1, pages 94-100, see entire document.	1, 3 ------ 2, 4
Y	US 5,698,399 A (DUFF ET AL) 16 December 1997, columns 3 and 4 and claim 1.	1-4
Y	GenCore database, N-Geneseq; ID No. Q10956. EP 417,563 A (HOFFMANN-LA ROCHE AG) 20 March 1991, see entire document.	2, 4
Y	GenCore database, N-Geneseq; ID No. Q10907. AU 9058976 A (SYNERGEN INC.) 24 January 1991, see entire document.	2, 4
A, P	US 5,948,638 A (LIN ET AL) 07 September 1999, column 1.	1-4

X	Further documents are listed in the continuation of Box C.		See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 MARCH 2000	0 6 APR 2000

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer BJ FORMAN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,766,585 A (EVANS ET AL) 16 June 1998, column 6.	1-4
A	US 5,837,832 A (CHEE ET AL) 17 November 1998, column 31.	1-4