Virus and host determinants of HIV-1 infection and AIDS pathogenesis
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Chapter 3

'Susceptibility of in vitro stimulated PBMC to infection with NSI HIV-1 is associated with levels of CCR5 expression and β-chemokine production'

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Coli with lower susceptibility. The genotype of an individual's genotype of HLA-A, B, C, DRB1 is important in the context of transplantation. However, the presence of an ILT-4 allele in the context of HIV-1 infection can alter the immune response to HIV-1 infection. Therefore, the combination of HLA and ILT-4 alleles is important in determining HIV-1 susceptibility.

Compared to the combination of HLA-B*40:01 and HLA-DRB1*09:01, the combination of HLA-B*57:01 and HLA-DRB1*01:01 leads to a reduced risk of HIV-1 infection. This is due to the presence of the HLA-B*57:01 allele, which is associated with a lower risk of disease progression in HIV-1 infection. However, the presence of the HLA-B*57:01 allele is not sufficient to completely prevent HIV-1 infection, as other factors such as viral load and immune response also play a role.

The results of this study suggest that the combination of HLA-B*57:01 and HLA-DRB1*01:01 is associated with a lower risk of HIV-1 infection compared to the combination of HLA-B*40:01 and HLA-DRB1*09:01. However, additional research is needed to fully understand the role of HLA and ILT-4 alleles in determining HIV-1 susceptibility.

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Susceptibility of in vitro stimulated PBMC to infection with NSI HIV-1 is associated with levels of CCR5 expression and β-chemokine production

Susceptibility of phytohemagglutin and recombinant interleukin-2 (PHA/rIL-2)-stimulated peripheral blood mononuclear cells (PBMC) from 14 healthy blood donors to non-syncytium-inducing (NSI) human immunodeficiency virus type 1 (HIV-1) infection was analyzed in relation to CCR5 expression and β-chemokine production. After 1 week of culture in the presence of rIL-2, but not at the moment of inoculation, CCR5 surface expression was positively and β-chemokine production inversely associated with susceptibility to NSI HIV-1 infection. Surprisingly, no association was observed between CCR5 genotype and in vitro NSI HIV-1 susceptibility, which was in agreement with similar levels of CCR5 surface expression and β-chemokine production in CCR5A32/+ and CCR5 +/− PBMC after PHA/rIL-2 stimulation. In contrast to what was observed in vitro, CCR5 genotype did associate with CCR5 surface expression levels in vivo in resting as well as in activated CD4+ T cell populations that were identified by the expression of CD45RO, CD27, HLA-DR and CD69. The association between CCR5 expression and susceptibility to infection by NSI HIV-1 in vitro, likely explains the in vivo observed protective effect of CCR5 polymorphisms that influence CCR5 expression on disease progression. Additionally, genetically based differences in the level of β-chemokine production may enhance the effect of CCR5 mutations and contribute to slow disease progression also in individuals with a wild-type CCR5 genotype.

Non-syncytium-inducing (NSI) variants of human immunodeficiency virus type-1 (HIV-1) use the β-chemokine receptor CCR5 as a cofactor for entry of target cells. The relevance of this receptor in HIV-1 infection and AIDS pathogenesis has become clear by virtue of genetic polymorphisms influencing its expression levels. In the human Caucasian population, the most prevalent polymorphism in the coding region of CCR5 is a 32-bp deletion (CCR5A32). Individuals homozygous for CCR5Δ32 (CCR5 Δ32/Δ32), who consequently lack CCR5 cell surface expression, are protected from HIV-1 infection. However, the existence of HIV-1-infected CCR5 Δ32/Δ32 individuals showed that this protection is not absolute. Yet, the presence of the natural ligands of CCR5: RANTES, MIP-la, and MIP-lβ, which might be explained by transmission of viruses that use CXCR4 as a coreceptor, was shown not to be protected from infection, but CCR5 Δ32 heterozygosity has been associated with a mean elongated AIDS-free survival period compared to individuals with the wild-type CCR5 genotype (CCR5 +/−). More recently, also other polymorphisms in the coding region and the promotor region of CCR5 have been shown to be associated with disease progression. In line with the in vivo observations, peripheral blood mononuclear cells (PBMC) from CCR5 Δ32/+ individuals were shown to be less susceptible to NSI HIV-1 in vitro and in vivo in PBL-SCID mice compared to PBMC from CCR5 +/+ individuals. The on average lower proportion of CCR5-expressing CD4+ T cells and lower CCR5 surface expression levels on PBMC, may result in slower spread of the virus and hence explain reduced NSI HIV-1 susceptibility of PBMC and slower disease progression in CCR5 Δ32/+ individuals.

An additional factor influencing HIV-1 replication is the presence of the natural ligands of CCR5: RANTES, MIP-1α, and MIP-1β. These ligands were shown to inhibit NSI replication in vitro, and high endogenous production by PBMC and/or CD4+ T lymphocytes was associated with protection from HIV-1 infection in vivo and protection from HIV-1 disease progression. The inhibitory effect of β-chemokines is proposed to act through blocking of the coreceptor as well as through down-regulation of CCR5 on the cell surface. The relevance of β-chemokine production in HIV-1 infection and AIDS pathogenesis has become clear by virtue of genetic polymorphisms influencing its expression levels.
disease progression was recently supported by the identification of a polymorphism in the RANTES promoter that associates with reduced RANTES secretion and concomitantly with reduced CD4⁺ T cell decline[298].

We here analyzed CCR5 expression levels and β-chemokine production of total PBMC and PBMC depleted of CD8⁺ T cells, after in vitro stimulation with PHA and rIL-2. In parallel, the susceptibility for a panel of primary NSI variants was determined and the relation with CCR5 and β-chemokine levels was studied.

**MATERIALS AND METHODS**

**Cells and viruses.** Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats from 14 healthy plasmapheresis donors by ficoll-paque isolation. PBMC were stored in liquid nitrogen until usage. Three days prior to infection (t=-3) thawed PBMC (5·10⁶/mL) were stimulated with phytohemagglutinin (PHA; 1µg/mL). At the day of infection (t=0), PHA-supplemented medium was removed and replaced by fresh rIL-2-supplemented medium. At day 7, 1/3 of the medium was removed and replaced by fresh rIL-2-supplemented medium. At day 14, virus production was determined with an in-house ELISA.

**Susceptibility assay.** Seven biological HIV-1 clones previously obtained from 3 different participants of the Amsterdam Cohort Studies on HIV-1 infection and AIDS (ACH). The NSI phenotype (as determined on the MT2 cell line[281]) were titrated on PHA-PBL (on average 67% of CD3⁺ T cells were CD4⁺ and 25% were CD8⁺) and CD8⁺ PHA-PBL (on average 93% of CD3⁺ T cells were CD4⁺ and 2% were CD8⁺) from each of the 14 blood donors. Briefly, the cells were inoculated with serial 5-fold dilutions of each virus stock and cultures were maintained for 14 days. At day 7, 1/3 of the medium was removed and replaced by fresh rIL-2-supplemented medium. At day 14, virus production was determined with an in-house ELISA[394] and the 50% tissue culture infectious dose (TCID₅₀) per mL of virus stock was determined. For each donor, the average TCID₅₀ of 7 NSI viruses was used as a measure of NSI HIV-1 susceptibility of their PBMC and CD8⁺PBMC.

**CCR5 genotyping.** Genomic DNA was isolated from fresh PBMC (Qiagen; Westburg, Hilden, Germany). CCR5 genotyping was performed by PCR analysis using primers flanking the 32-bp deletion in CCR5[395].

**FACS analysis.** Prior to stimulation (t=-3), after PHA stimulation, (i.e., just before inoculation; t=0), and after 6 days of mock-infection in the presence of rIL-2 (t=6), cells were stained with a combination of monoclonal antibodies (mAbs) directed against CD4 (-TC; Caltag, Burlingam, CA) and CCR5 (5G7-FITC; Pharmingen, San Diego, CA). Since after 1 week of culture the proportion of viable CD4⁺ T cells decreases rapidly (unpublished observation), and hence most of the viral spread through the culture will occur during the first week, no analysis was performed on cells during the second week of the culture period. To monitor the efficiency of CD8 depletion, cells were stained with a combination of mAbs directed against CD3 (-PE), CD8 (-FITC), and CD4 (-TC) (all mAbs from Caltag, Burlingam, CA).

In order to determine CCR5 expression levels on in vivo activated CD4⁺ T cells, cryopreserved, unstimulated PBMC were stained with a combination of 1: CD4 (-PERCP; Becton Dickinson, San Jose, CA), CD45RO (-APC; Becton Dickinson), HLA-DR (-PE; Caltag), and CCR5 (5G7-FITC; Pharmingen); 2: CD4 (-PERCP; Becton Dickinson), CD45RO (-PE: DAKO, Glostrup, Denmark), CD69 (-APC; Becton Dickinson), and CCR5 (5G7-FITC; Pharmingen); 3: CD4 (-PERCP; Becton Dickinson), CD45RO (-APC; Becton Dickinson), CD27 (-FITC; CLB, Amsterdam, Netherlands), and CCR5 (5G7-PE; Pharmingen). All incubation steps were performed for 20 min at 4°C. Expression of the markers was analyzed with a FACScan or a FACScalibur (both from Becton Dickinson).

**β-Chemokine production.** At t=0 and t=6, 200 µl of cell-free culture supernatant was sampled and stored at -20°C until analysis. The presence of MIP-1α, MIP-1β, and RANTES in the supernatant was determined by ELISA (R&D systems: Minneapolis, MN).

**Statistical analyses.** Comparisons between cells with the CCR5⁻/⁻ and CCR5 Δ32/⁻ genotype, between cells with high and low susceptibility, and between total PBMC and CD8⁺PBMC were made with the Student's t-test. In the cases of comparisons of β-chemokine levels, statistics were performed on the log-transformed values. The correlation between CCR5 expression and the log-transformed values of RANTES was determined with the Pearson's correlation coefficient (r). Normality of the samples was determined by the Shapiro-Wilk W-test for normality. All statistical analyses were performed by the use of SPSS 7.5 for windows.
Fig. 1. Susceptibility of total PBMC and CD8+ PBMC for NSI HIV-1. The TCID_{50} of 7 NSI viruses were determined by p24 production after 14 days of culture. For each donor, 7 CCR5 +/+ and 7 CCR5 Δ32/+ individuals, the average 10^log TCID_{50} on parallel cultured PBMC (●) and CD8 PBMC (△) are depicted. Bars represent the standard error of the mean. Differences were analyzed with the paired t-test and statistical significance is indicated by * (P<0.05) and † (0.05 < P < 0.06).

RESULTS

Susceptibility of total PBMC and CD8+ PBMC.

Three-day PHA-stimulated PBMC and CD8+PBMC derived from 14 healthy donors were inoculated with a panel of 7 primary NSI variants, and further cultured in the presence of rIL-2. The donors were selected based on their CCR5 genotype: 7 had the CCR5 Δ32/+ and 7 had the CCR5 +/+ genotype. After 14 days of culture, the TCID_{50} was determined for each virus-donor combination. Since cells from each donor were inoculated with the same stock of each virus, the differences in the virus titers are a reflection of differences in NSI HIV-1 susceptibility of the cells. Paired analysis of PBMC and CDR3PBMC showed that the susceptibility was similar in 11/14 cases (Fig. 1). For one donor the susceptibility of PBMC was higher than that of CD8+PBMC (donor 1: average TCID_{50}: 10^{4.60} versus 10^{4.22}, respectively; P = 0.028). For two donors the inverse was observed, with reduced susceptible PBMC compared to CDR3PBMC (donor 7: average TCID_{50}: 10^{4.09} versus 10^{4.39}, respectively; P = 0.058; donor 10: average TCID_{50}: 10^{4.40} versus 10^{4.25}, respectively; P = 0.002). Although inter-individual differences in susceptibility were observed, these differences were not related to differences in CCR5 genotype (Fig. 1).

Correlates of NSI HIV-1 susceptibility.

For the PBMC of each donor, the average TCID_{50} of 7 NSI variants was used as a measure for NSI HIV-1 susceptibility. In order to classify the relative NSI susceptibility of the PBMC from each of the 14 donors, the median of the 14 average TCID_{50} values was determined. PBMC with an average TCID_{50} below that of the group median were defined as having relatively low NSI HIV-1 susceptibility, PBMC with an average TCID_{50} equaling or above that of the group median were defined as having relatively high NSI HIV-1 susceptibility. Thus, 7 donors with less and 7 donors with more susceptible PBMC could be identified. For these 2 groups of donors, CCR5 expression prior to stimulation (t=-3) and CCR5 expression and B-chemokine production after 3 days of PHA stimulation (i.e., just prior to inoculation; t=0) were compared. In parallel cultures that were mock-infected, the CCR5 expression and B-chemokine production after further propagation in
In vitro susceptibility to NSI HIV-1 infection

Fig. 3. β-Chemokine production of cells with high and low susceptibility. MIP-la-, MIP-lß-, and RANTES-production in culture supernatants were compared for PBMC with high (G) and low (M) susceptibility. Analysis was performed on culture supernatants harvested after (a) 3 days of stimulation with PHA (t=0), and (b) again after 6 days of mock-infection in the presence of rIL-2 (t=6). Error bars represent the standard error of the mean. Differences were analyzed with the Student’s t Test on the log transformed values and statistical significance is indicated by * (P<0.05).

Similarly, at the end of 3-day PHA stimulation, no difference was observed in the mean production of MIP-la, MIP-lß, and RANTES between PBMC with relatively high and relatively low NSI HIV-1 susceptibility (Fig. 3a). After 6 days of mock-infection, the mean level of each β-chemokine was higher in the cell cultures with the lowest susceptibility (mean values: MIP-la: 10.6 versus 2.7 ng/ml; P= 0.025; MIP-lß: 5.5 versus 2.3 ng/ml; P = 0.042; RANTES: 6.4 versus 0.6 ng/ml; P = 0.010; Fig. 3b).

The percentage of CCR5+ cells and the mean fluorescence intensity (MFI_{CCR5}) in the CD4+ T cell population prior to stimulation and at the moment of inoculation did not differ between donors with relatively low and donors with relatively high in vitro NSI HIV-1 susceptibility (Fig. 2). However, in vitro susceptibility of PBMC was highly associated with the levels of CCR5 surface expression and β-chemokine production at t=6 (data not shown).

**Association between CCR5 surface expression and β-chemokine production.** Both high CCR5 surface expression and low β-chemokine production after PHA/rIL-2 stimulation (i.e., at t=6) were associated with high in vitro NSI HIV-1 susceptibility. Since it was shown previously that low CCR5 cell surface expression is associated with high β-chemokine levels\(^{(42)}\), which is likely due to internalization of the receptor after chemokine binding\(^{(42)}\), we analyzed the correlation between β-chemokine production and CCR5 expression, in relation to susceptibility. No (or a weak) correlation was observed between the

![Correlation between RANTES production, percentage CCR5-expressing CD4+ T cells, and susceptibility. The levels of RANTES production in PBMC culture supernatants and the percentages of CCR5-expressing CD4+ T cells measured after 6 days of mock-infection are depicted. Correlation analysis was performed with the Pearson’s correlation coefficient after log transformation of the levels of RANTES. For each donor HIV-1 susceptibility of the cells is depicted by the color of the symbols (○ = low; ● = high) and the number next to the symbol, representing the log TCID\text{50}.

Fig. 4. Correlation between RANTES production, percentage CCR5-expressing CD4+ T cells, and susceptibility. The levels of RANTES production in PBMC culture supernatants and the percentages of CCR5-expressing CD4+ T cells measured after 6 days of mock-infection are depicted. Correlation analysis was performed with the Pearson’s correlation coefficient after log transformation of the levels of RANTES. For each donor HIV-1 susceptibility of the cells is depicted by the color of the symbols (○ = low; ● = high) and the number next to the symbol, representing the log TCID\text{50}.
percentages of CCR5+ CD4+ T cells and the log-transformed values of levels MIP-1α and MIP-1β after PHA/rIL-2 stimulation \((r_s = -0.455, P = 0.10; \text{and } r_s = -0.301, P = 0.30, \text{respectively; not shown})\). A strong inverse correlation was observed however, for the percentages of CCR5-expressing CD4+ T cells and the level of RANTES production \((r_s = -0.630, P = 0.016; \text{Fig. 4})\). It is clear from Figure 4 that the PBMC with low susceptibility have both a relatively low proportion of CCR5-expressing CD4+ T cells and a relatively high level of RANTES production.

**CCR5 genotype and in vitro NSI HIV-1 susceptibility.** In contrast to what was found previously by some\(^{300, 405, 415, 416}\), but in agreement with others\(^{417}\), we did not observe that susceptibility to NSI HIV-1 infection in vitro was associated with the CCR5 genotype (Fig. 1). In order to understand the basis of this dissociation, we analyzed the association of CCR5 genotype with in vitro induced CCR5 surface expression and β-chemokine production after PHA/rIL-2 stimulation. As it was shown previously\(^{405, 418}\), the percentage of CCR5-expressing CD4+ T cells was associated with the CCR5 genotype prior to stimulation \((24\% \text{ in CCR5} +/+ \text{ and 14}\% \text{ in CCR5} \Delta 32/+; P = 0.006; \text{Fig. 5a})\). However, after PHA/rIL-2 stimulation this association was lost \((31\% \text{ CCR5-expressing CD4}^+ \text{ T cells both in CCR5} +/+ \text{ and CCR5} \Delta 32/+ \text{ PBMC}; P = 0.96; \text{Fig. 5b})\).

The similar CCR5 expression levels in CCR5 +/+ and CCR5 \Delta 32/+ cells after stimulation could be explained by an on average higher up-regulation in CCR5 \Delta 32/+ \((\text{on average: } +17\% ; \text{range: } +2 \text{ to } +40\%)\) compared to CCR5 +/+ \((\text{on average: } +7\% ; \text{range: } -16 \text{ to } +31\%)\) cells. Similar observations were made for CCR5 expression as measured by the MFI \((\text{not shown})\). In additional, no CCR5 genotype-related differences in β-chemokine production were observed after PHA/rIL-2 stimulation \((\text{mean values of 22.1 and 6.0 ng/ml for CCR5} +/+ \text{ and CCR5} \Delta 32/+ \text{ PBMC, respectively; } P = 0.13; \text{ Fig. 5c})\).

**CCR5 genotype and CCR5 surface expression on in vivo activated cells.** The absence of an association between CCR5 genotype and CCR5 expression after in vitro stimulation prompted us to study the association between CCR5 genotype and CCR5 surface expression on in vivo activated cells. To this purpose, CCR5 expression was analyzed on resting and activated CD4+ T cells in unstimulated PBMC from the 14 healthy blood donors. In vivo activation was identified by cellular expression of CD45RO in the absence of CD27 expression\(^{423, 424}\) or in combination with either HLA-DR or CD69 expression. The proportion of CCR5-expressing cells was very low within the naive CD4+ T cell population (average values:
In vitro susceptibility to NSI HIV-1 infection

38 In vitro susceptibility to NSI HIV-1 infection

108 memory activated memory

Fig. 6. Ex vivo CCR5 surface expression on resting and activated CD4+ T cell subsets. (a) The average percentage of CCR5-expressing cells in total CD4+ T cells (hatched bar) and in naive (D), resting memory (■), and activated memory (□) CD4+ T cells was determined. (b and c) For the resting CD4+ memory (b) and the activated CD4+ memory (c) T cells, the expression levels on CCR5 Δ32/+ and CCR5 +/+ PBMC were analyzed separately. The subsets were defined as indicated in the figures. Vertical bars (a) represent the standard error of the mean, and horizontal bars (b,c) indicate the average percentage of CCR5+ CD4+ T cells within each CD4+ T cell subset and the standard deviation. Differences were analyzed with the Student’s t-test.

CD45RO+/HLA-DR+: 7%, CD45RO+/CD69+: 3%; CD45RO-CD27+: 0.7%; Fig. 6a), and not statistically different between CCR5 +/+ and CCR5 Δ32/+ PBMC (not shown). The proportion of CCR5-expressing cells was highest in the activated memory CD4+ T cells (average values: CD45RO+/HLA-DR+: 50%, CD45RO+/CD69+: 62%, CD45RO-CD27+: 60%, Fig 6a), and intermediate in the resting memory CD4+ T cells (average values: CD45RO+/HLA-DR+: 29%, CD45RO+/CD69+: 25%, CD45RO-CD27+: 22%; Fig. 6a). The association between the level of CCR5 expression and activation status of the cells is in agreement with previous observations.

Both in the resting and in the activated memory CD4+ T cell populations, the proportion of CCR5-expressing cells (Fig. 6b and c) and CCR5 expression as measured by MFI (not shown) were on average lower in CCR5 Δ32/+ compared to CCR5 +/+ PBMC.

DISCUSSION

Susceptibility of PBMC and PBMC depleted of CD8+ T cells for in vitro NSI HIV-1 infection was associated with the expression of the coreceptor for NSI variants, CCR5, and the levels of its natural ligands, the β-chemokines, produced during culture. This association was only observed for 3-day PHA stimulated PBMC that had subsequently been cultured for 6 days in the presence of rIL-2, paralleling 6 days of infection. The absence of an association of HIV-1 susceptibility with CCR5 surface expression and β-chemokine levels immediately after 3-day PHA stimulation, so at the moment of inoculation, suggests that under the presently used conditions virus production is determined by the capacity of the virus to spread through culture, rather than by the efficiency of the initial inoculation.

In HIV-1-infected individuals in general, a correlation has been demonstrated between a CCR5Δ32 heterozygous genotype and a more benign clinical course of HIV-1 infection. This was attributed to a reduced percentage of CCR5-expressing cells, the target cells for NSI HIV-1. Indeed, early in infection the mean viral load in CCR5Δ32 heterozygotes was shown to be lower than in CCR5+/+ individuals, indicative of impaired virus replication. Here, we did not observe a correlation between CCR5 genotype and in vitro NSI HIV-1 susceptibility. This by itself was in
good agreement with the equal CCR5 surface expression in CCR5Δ32+/+ and CCR5+/+ PBMC after PHA/rIL-2 stimulation. The equal CCR5 surface expression in the two genotypic groups and the absent difference in NSI HIV-1 susceptibility may however be an in vitro artefact. Indeed, in the PBL-SCID mouse model, replication of NSI HIV-1 was significantly lower in mice repopulated with PBMC from a CCR5Δ32 heterozygous donor compared to mice repopulated with CCR5+/+ PBMC. Also, as we demonstrate here, ex vivo CCR5 expression is generally lower on cells from CCR5Δ32 heterozygotes, even on CD4+ T cells with an activated phenotype, that were activated in vivo under more physiological conditions. Although the CCR5 surface expression on cells from CCR5Δ32+/+ individuals is generally lower, there is a large overlap with the expression on cells from CCR5+/+ individuals. This indicates that the CCR5 surface expression is not only influenced by the CCR5Δ32 genotype but under control of multiple mechanisms. Indeed, polymorphisms in the CCR5 promoter region have been demonstrated that might influence expression levels. In addition, the natural ligands are able to down-modulate their receptor and high β-chemokine levels may consequently result in reduced CCR5 expression. In agreement with this is the inverse correlation we here observed between RANTES production and CCR5 surface expression. The down-modulation of CCR5 by RANTES may explain the association between β-chemokine production and reduced susceptibility for NSI HIV-1. In addition the β-chemokines may directly interfere with HIV-1 entry.

Our data indicate that the CCR5 expression level, and not CCR5 genotype perse, is an important determinant for NSI HIV-1 susceptibility in vitro. In agreement, we previously demonstrated that among individuals with a CCR5 +/+ genotype, a reduced CCR5 surface expression correlated with a better prognosis of HIV-1 infection. Even though CCR5 Δ32/+ individuals are generally not protected from infection, a higher frequency of CCR5Δ32+/+ individuals and reduced in vitro susceptibility to HIV-1 infection was observed in uninfected monogamous sexual partners of infected individuals. This suggests that reduced susceptibility may tip the balance in favor of the exposed individuals under certain conditions. In addition, susceptibility of cells is likely to be of importance during acute infection. Individuals that subsequently progressed to AIDS had already higher CCR5 surface expression levels pre-seroconversion compared with individuals that did not progress to AIDS in the same infection period. Thus, lower CCR5 surface expression and reduced susceptibility may result in a less severe viremia early in infection and consequently in a lower viral set-point, which is associated with a better prognosis.

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D-VIIH SEAH 12T mammalian transfected with BEH from a B-lymphoblastic cell line, and is used to express the SEAH 12T gene. The expression of SEAH 12T in vitro was assessed by Western blot analysis, which revealed the presence of SEAH 12T protein in the transfected cells. The expression of SEAH 12T in vivo was assessed by Southern blot analysis, which showed the presence of SEAH 12T DNA in the transfected cells. The expression of SEAH 12T in vivo was assessed by Northern blot analysis, which showed the presence of SEAH 12T mRNA in the transfected cells. The expression of SEAH 12T in vivo was assessed by in situ hybridization, which showed the presence of SEAH 12T RNA in the transfected cells.